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STUDIES ON IN VIVO REGULATION OF RIBONUCLEIC
ACID SYNTHESIS DURING INITIATION OF SPORULATION
IN BACILLUS SUBTILIS.

City University of New York, Ph.D., 1977
Biology

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1976

STUDIES ON IN VIVO REGULATION OF
RIBONUCLEIC ACID SYNTHESIS DURING
INITIATION OF SPORULATION IN
BACILLUS SUBTILIS

BY
DOUGLAS TESTA

A dissertation submitted to the Graduate Faculty
in Biology in partial fulfillment of the require-
ments for the degree of Doctor of Philosophy.
The City University of New York.

1976

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Studies On In Vivo Regulation of Ribonucleic Acid Synthesis During
Initiation of Sporulation in *Bacillus subtilis*

BY

Douglas Testa

Advisor: Professor Rivka Rudner

The process of sporulation in wild type (sp^+) and asporogenous (sp^-) mutants of *Bacillus subtilis* was studied in three media. The growth and sporulation related parameters assayed during morphogenesis demonstrated patterns that were specific to the developmental capacity of the cells studied. It was observed in cells in the Sterlini and Mandelstam (SM-R) media, that the net accumulations of nucleic acids and proteins stopped, and the sporulation-specific production of alkaline phosphatase and heat resistance first appeared at T_2 and T_8 respectively. Some cells capable of sporulating in the SM-R media (sp^+), when grown in the Sonenshein and Roscoe (121B) medium, did not produce alkaline phosphatase or heat resistant cells and therefore behaved as if they were metabolically blocked before stage T_2 . These cells are referred to as medium-induced asporogenous phenocopies (SpoM_C). One of these SpoM_C strains, A26u⁻, was observed

to be metabolically blocked at or before T_0 because of its inability to produce an extracellular serine protease activity which characteristically appears at T_0 in all media.

The rates of RNA synthesis in the sp⁺, sp⁻ and SpoM_C cells studied, was observed to radically decrease to levels 5-10% of the observed vegetative rate, during the first hour in stationary phase. This first hour in stationary phase is referred to as the transition period. The rate decrease did not occur in strain A26u⁻ in 121B medium. A rate decrease similar to the one described, was also observed in cells adjusting to a new carbon source.

The synthesis of ribosomal RNA at T_2 of sporulating and non-sporulating cells was assayed by the techniques of DNA-RNA hybridization and competition. All stationary phase cells demonstrated an approximate 50% decrease in the levels of rRNA synthesis compared to the levels observed in vegetative cells. Preincubation of the cells at T_2 with chloramphenicol (CM) was found to completely restore the rRNA content to vegetative levels in the asporogenous cells (sp⁻) and only partially raise the content of rRNA in sp⁺ and SpoM_C cells at that time, i.e. a 15% increase. In addition the drug (CM) increased the quantities of L-specific mRNAs made during vegetative growth and stationary phase of sp⁺ and SpoM_C cells. No change was observed in the quantity of L-mRNA synthesized at T_2 in asporogenous cells pretreated with chloramphenicol.

Segall et al., (1974) have proposed that CM prevents the synthesis of a short-lived inhibitor of the vegetative sigma subunit of RNA polymerase. The addition of chloramphenicol to SpoM_C cells in 121B medium showed the following: (1) only a partial restor-

ation of the vegetative levels of rRNA synthesis, a result similar to that found in the other sporulation media used also, and (2) no synthesis of new vegetative transcripts at T_2 as expected if the vegetative activity of the RNA polymerase was restored. Hybridization-competition experiments have also demonstrated that during stationary phase in sp^+ , sp^- and $SpoM_c$ cells, there is a decrease in rRNA synthesis and a quantitative increase in the mRNA specific to the MAK-fractionated DNA strands (designated L and H). In addition, both sporulating and $SpoM_c$ cells of A26u⁻ demonstrated a decrease in the transcription of vegetative genes during stationary phase and a "turn-on" of stationary phase-specific cistron transcription.

Analyses of the regulation of DNA synthesis during stationary phase in sporulating and non-sporulating cells revealed that sporulating cells do not continue to synthesize DNA. Non-sporulating cells, i.e. sp^- and $SpoM_c$ strains continue to re-initiate DNA replication. It was observed from these studies that the control over DNA synthesis and the rates of RNA synthesis were separately occurring events in sp^+ cells, and a scheme is presented to identify those events identified during the transition period, T_0 - T_1 , which are necessary for sporulation in Bacillus subtilis.

ACKNOWLEDGEMENTS

I will begin by conveying deep and loving appreciation to my wife, Rosemary, for her understanding and support throughout the course of my graduate study. I am extremely grateful to my research advisor, Professor Rivka Rudner, for her strong guidance, lasting friendship and criticism. Professors S. Anand Kumar, S. Marvin Friedman, Joseph Krakow and Constante Ceccarini have been helpful with critical conversations at various stages of this work.

A special thank you is made to other members of the Rudner laboratory who have spiritually contributed to the success of these studies. Most notable were Denise A. Galloway, Yoshiko Setoguchi, Saul Scheinbach, Virgilia Remeza, Joy Berman, Marie LeDoux and Lola Margulies. My sincere gratitude to Betty Ann Adorno for the typing of this manuscript.

To the countless number of other people that I have not listed who have been instrumental in the contributions made toward this work, I am truly grateful.

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PREFACE

The formation of spores from vegetative cells in Bacillus subtilis is a simple model system for cellular differentiation. the biochemical mechanisms which initiate and control the ordered cellular development may be identified in the less complicated bacterial cell. Sequential expression of specific genes permits an ordered programmed series of changes to occur within a cell which leads to the formation of an inactive cell, resistant to most environmental conditions. The temporal appearance of sporulation related enzyme activities leading to the differentiated spore is known to be the result of transcriptional and translational events. The period of development that has received a most intensive study but has eluded complete identification, is the transition period between vegetative growth and the advent of sporulation-specific events. It is during this period that the vegetative cell "recognizes" a change in the external environment and initiates changes in its internal metabolism. These changes prepare and direct the cell into the specific programmed metamorphosis which results in the cell becoming resistant to the external influences which would kill vegetative cells. In the introduction an attempt is made to identify the transition period during which initiation of sporulation occurs and to provide an understanding of the process of sporulation.

INTRODUCTION

The overall pattern of sporulation has been identified from studies correlating biochemical events with morphological changes observed in the electron microscope (Young and Wilson 1972) and is presented in Figure 1. Growing cells contain two distinct nucleoids which undergo a physical change into an axial filament during the first stage of sporulation. This change as well as the formation of an asymmetrically placed septum is reversible. The cell division resulting from the formation of the spore septum defines the smaller forespore and the mother cell. Engulfment of the forespore and continued differentiation results in the formation of a heat-resistant endospore. This programmed differentiation takes approximately 8-12 hours, depending upon the sporulation medium used. Mutants of Bacillus subtilis cells unable to sporulate (asporogenous) can be classified by the stage of development from which they do not pass, as they are identified biochemically and/or from electron photomicrographs.

Spore mutants have been used by investigators in hope of identifying a specific chromosomal location or episomal element which would carry the sporulation-specific genes. Hoch and Spizizen (1969) were successful in the identification of a tight cluster of genes in the bacterial chromosome which led to the expression of early sporulation-related events, namely the production of antibiotics and pro-

teases. This gene cluster appears to be under a common mechanism of control. Results from other laboratories revealed that other genes associated with sporulation are linked to markers on the B. subtilis chromosome, and are randomly distributed in gene clusters throughout the genome and not found on a detectable episome (Takahashi et al., 1965, 1969; Bott and Abelson, 1966). Young and Wilson (1972, 1975) using the available genetic data have assigned locations for these "spore" genes in relation to known B. subtilis genetic locations. A simplification of their map is presented in Figure 2. The indicated regions on the inner part of the circular B. subtilis map are the genes and gene clusters of known mapped sporulation mutants, many mutants remain to be identified (Piggot, 1973). Piggot (1973) and Hranueli et al. (1974) have referred to clusters of sporulation genes with their own "switch-on" mechanism and which deal with a specific stage of sporulation, as operons. The use of transduction and transformation experiments to map the sporulation genes, results in the identification of only 24 operons. The identification and characterization of new Spo mutants by Hranueli et al. (1974) demonstrated that 10 out of 16 mutants fell into known operons, whereas the remaining 6 fell into new unidentified operons. The authors conclude from this data, analyzed statistically, that the Spo operons identified to date represent only 60% of the probable number existing in the B. subtilis chromosome, i.e. 37 operons. The seven distinct morphological steps outlined in Figure 1 suggest that many operons are operative during each stage of

development. Coote and Mandelstam (1973) have suggested a difference between sporulation-associated and sporulation-dependent events which deal directly with spore formation. Sporulation specific events are those processes in sporulating cells which must occur in order for later development to follow. Sporulation related events occur along with sporulation specific events but do not themselves direct the developmental sequence. Dipicolinic acid found only in spores and sporulating cells but completely absent from vegetative cells, is an example of a sporulation-associated event since dipicolinic acid-less mutants of B. cereus can form viable spores (Wise et al. 1967).

The work of Spotts and Szulmajster (1962) with wild-type and asporogenous cells of B. subtilis suggested that there was a difference in the ribonucleic acid (RNA) transcripts in these cells as seen from sucrose density gradient analyses. Doi (1965) and Aronson (1965) working with two different bacilli, showed that there was a synthesis of sporulation-specific messenger RNAs (mRNA) during morphogenesis. This was later supported by Yamagishi and Takahashi (1968) in a comparison between sporulating and nonsporulating cell transcripts by DNA-RNA competition and hybridization analyses. It was obvious from these studies that sporulation was regulated at the level of transcription. Doi and his coworkers (Doi and Igarashi, 1964; Yamakawa and Doi, 1971; Sumida-Yasumoto and Doi, 1974) and DiCioccio and Strauss (1971, 1973) have clearly demonstrated in a series of DNA-RNA hybridization competition experiments, that genes expressed during sporulation were preferentially transcribed from the light (L-) DNA strand in addition to the transcription from the heavy (H-) DNA strand of B.

subtilis DNA. In addition, some vegetative genes were expressed during sporulation, although not all, and these are transcribed in the developing forespore (Jeng and Doi, 1974). Those vegetative genes not expressed during sporulation have been shown to be "turned-off" in a progressive manner as morphogenesis proceeds (Sumida-Yasumoto and Doi, 1974). Due to the close involvement of the forespore and the mother cell, it is not yet possible to totally identify those functions which may be specific to each cell.

Control of Transcription: Role of the DNA

Successful spore formation as mentioned, depends upon the messenger RNA species synthesized at the defined periods during development. Shaeffer, Millet and Aubert (1965) have suggested that induction of sporulation occurred through catabolite derepression, which was maintained during vegetative growth by an easily metabolizable nitrogen compound. This suggestion was supported by the appearance of new enzymatic activities not present in vegetative cells, i.e. serine protease, alkaline phosphatase and glucose dehydrogenase. Sterlini and Mandelstam (1969) have shown that when transcription is blocked in the sporulating cell, appearance of these sporulation-related proteins is blocked, again implying that their synthesis is induced at specific times during development.

Regulatory genes in the DNA, e.g. promoters and operators, serve as functional signals for proteins which bind to their DNA sequences and determine the fate of transcription of the adjacent cistrons within the operon. Szybalski et al. (1966) suggest that pyrimidine clusters may serve as recognition or initiation sites for RNA polymerase, since these clusters have been found only in the transcribed strand of coliphage T7. Experiments in E. coli (Rudner and LeDoux, 1974) and in B. subtilis (Rudner et. al., 1972) identify the presence of these pyrimidine clusters in both the L- and H- DNA strands. The authors suggest that these clusters may serve as the signals for transcription since the frequency of these sequences can be positively correlated with the extent of asymmetric transcription. The largest oligonucleotide lengths found in B. subtilis i.e 7-11 oligodeoxynucleotides, occur predominantly in the H- DNA strand which is to code for approximately 80% of the mRNA transcripts in the cell. Interestingly, there appears to be many more pyrimidine clusters on the L- DNA strand than is expected for a strand that is known to only code for approximately 20% of the mRNA species made. It is quite possible that there is a functional difference between these clusters and that they may serve as regulatory sequences only at specific times in the life cycle of the bacillus. This is supported by the report of Yamakawa and Doi (1971) who have shown that there is a preferential transcription of the L- DNA strand during sporulation.

Intrastrand secondary structure in DNA is observed in B. subtilis (Rudner and Remeza, 1973; Galloway, 1975) and in E. coli (Kato et. al., 1974) and is believed to comprise 5-10% and 2% of the respective bacterial genomes. If these secondary structural conformations do influence specific protein interactions with the DNA, then the formation or removal of these structures may serve as the in vivo control signals mediating many changing processes. The rate of initiation of RNA synthesis at the promoter is believed to be influenced by the supercoiling of the DNA or proteins binding to the promoter, (Travers, 1974). A repressor or negative control element would prevent the favored promoter conformation whereas a positive control element would enhance the favored conformation for RNA polymerase initiation, i.e. CRP (deCrombrughe et al., 1971b; Anderson et al., 1972). The conformational states of the DNA can be influenced by regulatory proteins which determine the frequency of initiation of new RNA chains from the promoter, by RNA polymerase.

Sporulation, as described, proceeds by an ordered series of transcriptional processes, obviously very carefully controlled for correct development. In addition to the possible changes in the transcription apparatus, i.e. RNA polymerase, which will be discussed later, structural differences may exist among the regulatory sites of the DNA. Bott and Abelson, (1966) have shown that the addition of acridine orange, a dye which intercalates in the DNA, to vegetative cells will result in the production of asporogenous mutants of B. subtilis. However, all the mutants identified were blocked after stage II of sporulation, i.e. no spo0 or spoI mutants. Similar findings were reported by Rogolsky and Nakamura (1974) using ethidium bromide, another intercalating dye. All asporogenous mutants were capable

of performing early spore functions, i.e. production of antibiotics and proteases, and were found to be blocked at or after stage II. These experiments strongly suggest that a physical difference between early and late sporulation genes and/or their control regions exists. Early sporulation and transition phase genes may share structural similarities to vegetative genes whereas sporulation genes functioning beyond stage II are physically distinct.

Control of Transcription: Role of the RNA Polymerase

The control of gene expression in developing bacterial and bacteriophage systems has been dominated by the study of RNA polymerase and the molecules which affect its activity (Bautz, 1972; Losick; 1972). E. coli RNA polymerase holoenzyme is a large protein assembly composed of multiple subunits, i.e. α_2 , β , β' , σ . Without the σ subunit the protein is referred to as the core enzyme (E) which receives its promoter site initiation specificity and efficiency from the σ (γ) subunit (Burgess, Burgess et al., 1969; Krakow et al., 1969). A similar enzyme has been isolated and identified in B. subtilis and its transcriptional role appears similar to the E. coli holoenzyme (Losick and Sonenshein, 1969; Shorestein and Losick, 1973a,b). As shown with E. coli (Haselkorn et al., 1969; Summers and Siegel, 1969) the B. subtilis RNA polymerase is required for the development of some bacterial phages (Geiduschek and Sklar, 1969). Although the rifampicin sensitive subunit of the host RNA polymerase is required throughout the growth cycle of coliphage T4 and B. subtilis phage SP01, coliphage T7 appears to require the host polymerase for transcription of only

early genes. In addition to using the host polymerase, modifications of the enzyme (Goff and Weber, 1970; Seifert et al., 1971) and association of new factors with the polymerase (Stevens, 1972) are reported during phage development. The transcriptional control exerted by the bacteriophage on the host enzyme lends another dimension to the approaches which may be used by a developing system for the control of gene expression.

Bacterial sporulation has been under intensive study during the past decade and the main objective is to uncover the molecular mechanisms involved in the programmed expression of the genome. Some of the most notable contributions in the area of transcriptional controls during sporulation have been made by Losick and his collaborators. Sonenshein and Roscoe (1969) showed that sporulating B. subtilis cells did not support the growth of ϕ e, a bacterial phage that multiplies in vegetative Bacillus subtilis cells. They proposed that the same transcriptional alterations occurring in sporulating cells which result in "turning off" of some vegetative cell functions were preventing the expression of ϕ e. Losick and Sonenshein (1969) demonstrated the coordinate decrease of development of ϕ e in vivo and the in vitro transcription of ϕ e DNA with stationary phase B. subtilis RNA polymerase early in the stationary phase. It was concluded from these experiments that the inability for phage multiplication in sporulating cells was due to a template specificity change in the RNA polymerase occurring early in sporulation. Additional work presented by Losick and colleagues (1970) demonstrated a difference in the subunit structure of the sporulation

RNA polymerase isolated from cells during the sixth hour of stationary phase, i.e. T_6 , when compared to the vegetative cells enzyme. This difference was observed as the absence of the sigma subunit and the modification of one of the beta subunits which appeared in a lower molecular weight form. The latter observation has since been shown to be an artifact of the polymerase isolation procedure (Orrego et al., 1973; Linn et al., 1973) and not observed when inhibitors of proteases are included in the sporulating cell extracts. This modification of the beta subunit, although first observed after T_2 of sporulation has been the focal point for theories on the mechanism on initiation of sporulation (Doi and Leighton, 1972). In addition, a series of experiments by Leighton et. al, (1971, 1972, 1973) identified that those cells which were unable to produce a functional serine protease during sporulation were also asporogenous and did not contain a modified beta subunit. The beta subunit modification is therefore accomplished by the action of serine protease induced at the onset of sporulation.

Still remaining unresolved is the event which leads to the template specificity change as observed with ϕ e, early in the sporulation sequence. Reports showed that some mutants resistant to the drug rifampicin (rfm^r) were also asporogenous or formed abnormal spores (Sonenshein et al., 1974; Santo et al., 1973A). This supported the involvement of RNA polymerase during development of the spore. Current reports altogether question the use of bacteriophage ϕ e as an indicator of the initial template specificity changes in RNA polymerase because these changes (1) are not always observed (Orrego, et al.,

1973), (2) appear to be dependent upon the sporulation medium used (Murray et al., 1974). Brevet and Sonenshein (1972) working with early blocked mutants found that the σ^E template specificity change was absent in all asporogenous cells blocked before stage II. The inability of the sigma subunit to co-purify with the sporulation polymerase was found to correlate with this change in template specificity (Brevet, 1974) and occurred early in stationary phase. In contrast to these findings Duie et al. (1974) using a sensitive immunoprecipitation assay, identified a sigma activity which was associated with the core enzyme, present in the sporulating and nonsporulating cells. Sonenshein et al. (1974) used a detailed genetic study to categorize rifampicin resistant and streptolydigin resistant B. subtilis cells with respect to their ability to sporulate in the presence of these drugs. The majority of the drug-resistant cells were capable of sporulating, however a minor mutant class (II) which was drug-resistant could not sporulate in the presence or absence of the drug. It is argued that the presence of this class of mutants lends credence to the hypothesis of "control by (RNA) polymerase" for sporulation. It appears that the decrease in the σ^E -directed transcription and the loss of the sigma-associated activity early during stationary phase could be the result of slowed growth found during this period. However, slower growth may not solely be responsible since stage 0 sporulation mutants were unable to inhibit the vegetative sigma-directed activities (Brevet and Sonenshein, 1972). The activity changes associated with RNA polymerase at the onset of sporulation may

fore be regarded as transition period changes. It should be recalled that sporulating cells still transcribe many vegetative genes (Sumida-Yasumoto and Doi, 1974; Yamakawa and Doi, 1971) although the overall rate of RNA synthesis is very much reduced.

It is generally accepted that RNA polymerase plays a regulatory role in sporulating cells, however it is not clear what that function is at the onset of stationary phase. If the vegetative sigma factor is only weakly associated with the core enzyme, what then is directing the sequence of events during the first few hours during sporulation (transition phase)? Changes in the proteins which bind the DNA have been associated with sporulation (Rhaese et al, 1974b). Greenleaf et al (1973 a, b) have identified a 70,000 dalton polypeptide associated with the core polymerase in sporulating cells. This protein is absent in spore mutants with a genetic defect prior to stage II of sporulation. This tryptophan -rich polypeptide appears between the first and second hours of stationary phase ($T_1 - T_2$), but its function has not yet been identified. This protein could be a new sigma factor replacing the vegetative sigma activity. Segall et al. (1974) have indirectly identified the presence of an unstable inhibitor of the vegetative sigma present in sporulating cells. The inhibitor is believed to decrease the association between the vegetative sigma factor and the core enzyme (Tjian and Losick, 1974). Treatment of sporulating cells with chloramphenicol (CM) restores sigma binding to the core polymerase, suggesting that synthesis of the inhibitor is linked to protein synthesis. Other

polypeptides associated with the sporulation core polymerase have been identified by Fukada et al., (1975). Two low molecular weight polypeptides, delta I, 27,000 daltons and delta II, 20,000 daltons, are found associated with the core polymerase at $T_{3.5}$ and $T_{5.5}$ respectively. They appear in a 1:1 stoichiometric ratio with the two beta polypeptides. The authors suggest that these delta polypeptides are necessary for the recognition of spore-specific promoters on the B. subtilis chromosome. This view is supported by the results of Santo et al. (1973a, b) which identify sporulation defective RNA polymerase mutants which are blocked between stages III and IV. This suggests that if the necessary change in the RNA polymerase is prevented, the cells can not proceed beyond that point in development.

Common to eucaryotic cell systems, is the presence of more than one type of RNA polymerase within the cell, each with its own template specificity (Roeder and Rutter, 1969; Chambon et al., 1969). More than one RNA polymerase activity has also been identified in E. coli by Travers and Buckland (1973). The bacterial enzymes of both E. coli and B. subtilis appear to exhibit differential activities for stable rRNA synthesis. Elongation factors EF Tu-Ts are asserted to be found associated with one of the polymerase activities which actively synthesizes stable RNA and in vitro this activity can be inhibited by the addition of the magic spot nucleotide MS I (ppGpp). These investigators have also identified a heterogeneous fraction of polymerase activity, which lacks the specificity for the rRNA cistrons and therefore may be involved in the transcription of the

messenger RNA fractions in vivo. It is of interest to note that stationary phase cells of E. coli, which synthesize less ribosomal RNA, lack the corresponding polymerase activity but contain non-transcribing polymerase molecules which may be complexed with regulatory molecules (Travers and Buckland, 1973).

Two RNA polymerase activities, i.e. I and II, have also been identified in Bacillus subtilis cells during vegetative growth (Diaz et al., 1975), both of which retain the rifampicin-sensitive subunit. Polymerase fraction I activity is three times greater than fraction II. Unfortunately, although the authors were able to demonstrate that EF Tu-Ts complex is associated with polymerase I, they were not able to show a preference of this enzyme for the rRNA cistrons over that observed with fraction II. During stationary phase, the activity of polymerase II decreased to a level which was only 15% of the vegetative activity. This activity decrease seems to be associated with sporulation since the stage 0 mutants tested did not show this change even by T₄. The demonstration of at least two different polymerase activities in bacterial cells and the decreased activity of one of these during the onset of the transition period implies that the turn-off of some vegetative genes during this time is due to the decreased activity of a vegetative-specific polymerase. Activity preferences of the RNA polymerase enzymes, with respect to transcription of the ribosomal cistrons, as shown by Travers and Buckland (1973) and as suggested by Diaz et al., (1975), implies that control of stable RNA synthesis may not actually be due to a sporulation-specific process, but rather the outcome of a slowed growth pattern.

Control Of Stable RNA Synthesis

Hussey, Losick and Sonenshein (1971) reported that early in the sporulation sequence of events, ribosomal RNA synthesis is "turned-off" and that this behavior is not observed in non-sporulating cells. Previous reports by Spotts and Szulmajster (1962) and Blassa (1963) demonstrated that stable RNA synthesis continued during sporulation, in addition to general RNA turnover. These events were found to occur even though the net accumulation of bulk RNA had stopped. Bonamy, Hirshbein and Szulmajster (1973) refuted the findings of Hussey et al. (1971). In similar experiments e.g. sucrose gradient analyses of incorporation of radioactivity into ribosomal RNA subunits, using a variety of B. subtilis strains, these investigators observed only a 30-50% reduction in the rRNA transcription during stationary phase of both sporulating and non-sporulating cells. This "turn-off" was not observed in another investigation by Testa and Rudner (1975a,b) who suggest that the decreased synthetic rate may be due to a naturally occurring step-down condition as is observed in stringent and relaxed bacteria, i.e. carbon source shift-down or amino acid starvation, also suggested by Pero et al. (1975).

One of the best studied systems of gene regulation has been the control of stable RNA synthesis (Maaløe and Kjeldgaard 1966; Edlin and Broda, 1968) during transient states of amino acid starvation or glucose step-down either induced artificially or naturally occurring (stationary phase). The stringent response was identified as the coordinate decrease in the rates of RNA and protein syntheses in bacteria auxotrophic for a specific amino acid, when starved for that amino acid. The mechanism of stringent control appears to be due to

the function of a product from the RC (rel) gene (Stent and Brenner, 1961) since relaxed mutants lacking the response have been identified (Swanton and Edlin, 1972). In bacteria the stringent response is correlated with the appearance of guanosine tetra- and penta-phosphates, Magic Spot I (MS I) and Magic Spot II (MS II) respectively (Cashel and Gallant, 1969; Cashel, 1969; Rhaese and Groscruth, 1974). These phosphorylated nucleotides have been synthesized in vitro by vegetative ribosomes associated with messenger RNA and occupied by an uncharged transfer RNA molecule in a tetracycline-sensitive site (Kaplan et al. 1973). The stringent response is controlled by the product of the RC (rel) locus and manifests itself as the decreased accumulation of stable RNA components. The rRNA that is synthesized has been shown to be in the unprocessed precursor form (Chang and Irr, 1973). Amino acid starvation has also been shown to have either stimulatory or inhibitory effects on messenger RNA transcription (Gallant and Margson, 1972; Lavelle and DeHauwer, 1968; Reiness et al., 1975). Reiness et al. (1975) show that ppGpp (MSI) can directly inhibit rRNA transcription in vitro possibly by the direct interaction with the bacterial RNA polymerase. To date, the appearance of the magic spot nucleotides (MSI and MSII), the involvement of an uncharged tRNA idling in the A site of the ribosome and the activity of the rel gene product have not completely explained the in vivo "turn-off" of rRNA transcription.

Synthesis of stable RNA components from a bacterial genome occurs in vivo with a higher transcription frequency than that for a specific messenger RNA. If it can be assumed that no great differences exist between the RNA polymerase core molecules used in the

transcriptional processes, then auxillary factors which act on the enzyme and/or the DNA at the promoter site may be invoked. This does not exclude the possibility that stable RNA promoters are structurally more available and conformationally more efficient for the initiation of transcription, ie. open promoters (Travers et al. 1973; Udvardy et al, 1974) since preferential synthesis of the ribosomal cistrons has been observed in vitro in the absence of any detectable stimulatory factors other than the sigma factor (Haseltine, 1972; Hussey, Pero, Shorestein and Losick, 1972). The quantity of rRNA synthesized in vitro however is not as large as observed in vivo, which therefore suggests that some stimulatory factors may be responsible for the increased rate of in vivo initiations. Travers, Kamen and Cashel (1970) postulated the existence of a protein factor psi-r, as a positive stimulatory factor specific to the ribosomal cistrons, which could be repressed by the action of the MSI and MSII nucleotides in some fashion. This appeared to be a reasonable answer to the problem concerning the stringent response until Haseltine (1972) showed that the E. coli RNA polymerase holoenzyme was just as efficient in rRNA transscription in vitro as it was with the psi factor, whether MS nucleotides were present or not. Travers (1973) has shown that the EF Tu-Ts complex, now equated with the psi factor, and a closed promoter configuration are the necessary prerequisites for stimulation of r RNA transcription, can be inhibited by the addition of MS nucleotides. It is very possible that under the conditions empolyed by Haseltine (1972) open promoters were available to the polymerase holoenzyme and therefore the reaction was independent

of EF Tu-Ts stimulation or ppGpp inhibition.

Protein synthesis translocation factor G (EFG) has also been implicated in the control of rRNA and MS nucleotide synthesis (Rabbini and Srinivasan, 1973). No ppGpp synthesis was observed during amino acid starvation in E. coli grown at the nonpermissive temperature when the cell is carrying a temperature-sensitive lesion in the gene coding for the EFG factor. Somehow EFG is involved in the rate of rRNA synthesis during balanced growth and the repression of rRNA synthesis during the stringent response (Kimura, Muto and Osawa, 1974). The synthesis of EFG and EF-Tu are now known to be under stringent control in E. coli (Furano and Wittel, 1976). Interestingly, the alpha subunit of RNA polymerase also appears to be under the stringent control system as do the beta-beta' subunits although the alpha and beta subunits seem to be autonomously regulated (Burgess, personal communication).

EF Tu has been shown to be copurify with one RNA polymerase activity (Diaz et. al., 1975) and MSI can directly inhibit the synthesis of rRNA in vitro (Reiness et al., 1975). These experiments indirectly suggest that the MSI nucleotide may inhibit the action of EF Tu stimulation. Jaskunas et al. (1975) have recently identified the presence of two copies for the EF Tu cistron in E. coli, one situated near a ribosomal protein cluster and the other linked to the rif gene. This evidence implies that the EF Tu-associated functions of protein and RNA synthesis may be mediated in vivo by two functionally distinct but structurally similar proteins.

Carbon source step down, like amino acid deprivation, also affects

the rate of ribosomal RNA synthesis in bacteria (Maaløe and Kjeldgaard, 1966; Neidhardt, 1963). The regulation of RNA synthesis during this carbon source step-down appears to be mediated by a mechanism differing from that observed in amino acid starvation, since relaxed cell mutants can still vary their rRNA synthetic rates with different growth rates (Neidhardt, 1963). Recently Laffler and Gallant (1974) have demonstrated that the product of the Spo T locus in E. coli is necessary for the conversion of ppGpp to pppGpp. The regulation of this conversion has been implicated in the regulation of RNA synthesis in response to shifts in the carbon and energy sources (Gallant et al., 1972). The stringent response may involve the increased rate of MSI synthesis and carbon source shift down reduces the turnover of MSI into MSII. This decreased turnover of MS nucleotides may be a result of the decreased availability of an intermediate of the glycolytic pathway that regulates the phosphorylation of MSI to MSII (Staminger and Lazzarini, 1974). Cashel and Gallant (1975) have proposed a model for the regulation of stable RNA synthesis. The rel gene product, located on the ribosome, is involved with the synthesis of MSI. This synthesis is controlled by a tetracycline-sensitive step involving the idling of an uncharged tRNA in the ribosomal A site. A second control mechanism involves the phosphorylation of ppGpp into pppGpp, which is mediated by the product of the Spo T gene and is coupled to the energy source during metabolism. The overall cellular concentration of MSI is controlled by the rate of its synthesis or its

disappearance into MSII. The quantity of this nucleotide (MSI) in the cell may therefore determine the number of RNA polymerase molecules with the specificity for initiation from rRNA promoters.

Control of Translation

The appearance of new proteins during sporulation due to the turn-on of new gene functions, may well be a result of selective translational discrimination for sporulation-specific messengers. Variations in the composition of ribosomal proteins have been observed in E. coli at different growth rates (Deusser and Wittman, 1972; Deusser, 1972). A population of ribosomes in sporulating cells may therefore contain factors which differ from the vegetative ribosomes also within the cell. The stable rRNA of sporulating cells, spores and vegetative cells has been shown to be identical in base composition and sequence as determined by hybridization and competition experiments (Bishop and Doi, 1968; Doi, 1965; Edgell et al., 1975; Testa and Rudner, 1975). Therefore any differential selective ability of ribosomes must reside in the protein composition of the sporulating cells. A difference in the resistance to fusidic acid observed in sporulating cell ribosomes by Fortnagel and Bergmann (1973) was not supported by later investigations (Guha and Szulmajster 1974; Fortnagel et al., 1975), and a new protein associated with the ribosome at T_0 appears to be the result of nutrient depletion of the medium. Domoto et al., (1975) and Leighton (1974) have demonstrated changes in the properties of erythromycin and streptomycin resistant B. subtilis

mutants associated with sporulation. These changes occurring by stage I-II of sporulation suggest the involvement of some translational control mechanisms during development.

Vold (1973), Bishop et al. (1969) and Chuang and Doi (1972) have correlated changes in the relative abundancies of specific transfer RNA populations, i.e. lysyl tRNA, during sporulation. Ribosomal involvement in sporulation may also be the result of slower growing cells as observed in E. coli (Deusser and Wittman, 1972; Deusser, 1972).

Control of DNA Replication

The DNA molecules in vegetative cells are compact and attached to the B. subtilis mesosome which is located at the position of the developing cell division septum (Mandelstam, Kay and Hranueli, 1975). During the first few hours in stationary phase, the DNA molecules form an axial filament and the on-going round of DNA synthesis is completed without reinitiation of new rounds (Sadoff, 1973). The halt in DNA synthesis is a precondition to the dormant state of the spore but the mechanism of termination of the process is not known. DNA synthesis may be stopped by repressing the synthesis of an initiator protein (Lark, 1969) or the direct inhibition of DNA synthesis by the antibiotics produced by the cells early in the stationary phase. Edeine, a peptide antibiotic produced early in sporulation by Bacillus brevis Vm4, will stop DNA synthesis in E. coli but will have no effect on the producer strain (Sadoff, 1972). The origin of this antibiotic and some of the other compounds identified, is not completely understood, however it is

known that RNA and protein synthesis is not required for their production. It is possible that these peptides 6-15 amino acids in length, are ~~cleavage~~ cleavage products from pre-existing proteins, cleaved by the proteases also induced at the end of vegetative growth (Kawamura and Ito, 1975). The production of these antibiotics is in some way tied into the process of sporulation since mutants defective in their production are also asporogenous.

Final completion of DNA synthesis therefore supplied the developing system with a full genetic complement which will be incorporated into the endospore. The induction of sporulation which prevents DNA initiation, may arise from the movement of the replicating fork through a stage 0 operon and therefore temporarily relieve this operon from repression. Mandelstam et. al (1971, 1974) used temperature sensitive DNA initiation mutants to demonstrate the appearance of a maximum capacity of the cells to be induced to sporulate. The peaks exist at a point on the chromosome some 15 minutes after replication has begun. This time corresponds to approximately 1/3 of the chromosome having been replicated under their experimental conditions. These investigators postulate that the replication fork (s) relieves the repressed state of an early spore operon located near the cys A chromosomal region (Mandelstam and Higgs, 1974) which if induced, "turns-off" new rounds of DNA synthesis.

In bacilli the chromosome, when visualized under the electron microscope, is associated with highly elaborate internal membranous structures called mesosomes. Mandelstam et al (1975) have

demonstrated that directly after axial filament formation during stationary phase, there is an association of the DNA with newly formed mesosomes located at the poles of the cell. The possibility exists that initiation of DNA synthesis is restricted to association of the chromosome with the median mesosome, which may be functionally different in its ability to foster initiation of DNA synthesis. Therefore new rounds of DNA synthesis can be prevented by the disassociation of the chromosome from a specialized mesosome. This hypothesis is supported by the work of Yamaguchi and Yoshikawa (1975) who show that both the origin (pur A) and the terminus (met B through thy A) of replication are associated with the B. subtilis membranes fractions (see Figure 2).

Initiation of Sporulation

Initiation of sporulation or the "triggering" event (s) that causes a cell to begin metamorphosis is the primary regulatory mechanism for spore formation. This period, which is synonymous with the transition period, is not to be confused with commitment, which is the time at which the biochemical and morphological events are irreversibly channeled toward the differentiated state (Sterlini and Mandelstam, 1969; Szulmajster, 1973). Commitment with regard to glucose utilization occurs at T_0 (Freese et al., 1969) and is observed as the inability of glucose

to prevent sporulation after this time. Commitment to other characteristic changes occurring during sporulation has been well documented by Sterlini and Mandelstam (1969) and are found to be scattered through development and not clustered at one stage. Hydrolyzed casein has the unique ability to prevent sporulation and stimulate growth at any time during sporulation prior to T_5 . These commitment times and the noted changes in the RNA polymerase already mentioned strongly suggest that the first few hours in stationary phase e.g. $T_0 - T_2$, represent a reversible uncommitted period which shares a great deal of characteristics with cells undergoing a transition to a slower growth rate, i.e. transition period, (Maaløe and Kjeldgaard, 1966; Edlin and Broda, 1968; Blassa, 1963; Spotts and Szulmajster, 1962; Testa and Rudner, 1975). During this transition period, the appearance of new molecules not present in vegetative cells, as a result of catabolic derepression (Shaeffer et al. 1965) may act to check the growth of the cells until the necessary nutrients are again present in the environment. Catabolic repression in bacilli however, does not appear to be under the same controls identified in E. coli since cyclic AMP has not been isolated from the bacilli (Flechtner and Hanson, 1969; Setlow, 1973).

Rhaese and his coworkers (Rhaese and Groscurth, 1974; Rhaese et al., 1972; Rhaese et al., 1975; Rhaese et al., 1976) isolated four highly phosphorylated nucleotides (HPN) in addition to MSI and MSII. These nucleotides HPNI, HPNII, HPNIII and HPNIV

have been identified as 5' ppApp3', 5' pppApp3', ppXpUpp and pppAppp respectively. Both the HPN and MS nucleotides are observed to increase in concentration approximately 30-60 minutes prior to T_0 in a nutrient sporulation medium and neither HPNI nor II are synthesized in response to an amino acid or glucose shift down (Rhaese et al. 1972). MSI and II are synthesized by the vegetative ribosomes isolated prior to T_2 (Rhaese and Groscurth, 1974) whereas HPNI and II are synthesized by sporulating cell ribosomes that contain the necessary protein(s) for their synthesis (Rhaese et al., 1975). The MS nucleotides do not play a crucial role in sporulating cells since relaxed mutants unable to synthesize these compounds are able to sporulate. It appears that the accumulation of the MS nucleotides in B. subtilis as with E. coli, is due to the stationary phase or shift-down conditions. The involvement of the HPN nucleotides in the initiation of sporulation has recently been put forth as a model by Rhaese et al., (1976). These investigators suggest that the external environmental stimulus is recognized by HPNIV or the enzyme that synthesizes HPNIV, which is located in the cell membrane, and by a complex series of events induces the internal change(s) leading to formation of the spore. No mutants deficient in HPN synthesis have been reported to date. It would be of great interest to identify the missing early events in the stationary phase of such mutants and if any or all of the HPN molecules are missing but required for initiation of sporulation. The results of such studies will more clearly separate sporu-

lation-specific from sporulation related events occurring during the initiation of morphogenesis.

The transition period therefore is a temporary stage during which the cell prepares to undergo or initiate morphogenic changes but still maintains the potential to return to vegetative growth should the environmental conditions change. The early stationary phase appearance of phosphorylated nucleotides e.g. MS and HPN, correlates well with the disassociation of the vegetative sigma subunit from the core polymerase. Segall et al. (1974) have reported that an inhibitor of sigma appears early in the sporulation sequence and that the synthesis of this inhibitor is blocked by chloramphenicol (CM). It would not be surprising if this inhibitor is identified as one of the MS or HPN nucleotides, since chloramphenicol has been shown to antagonize the stringent response (Kurland and Maaløe, 1962) and block the production of ppGpp (MSI) (Cashel, 1969). The template specificity change may result from the reduced metabolism of stationary phase cells which mimics starvation conditions. These nutrient poor conditions in bacilli are also known to stimulate the production of nucleases and proteases at T_0 (Kawamura and Ito, 1975) which may be involved in replenishing depleted internal pools that must be maintained at certain minimum levels for vegetative growth. Antibiotic accumulation, which is independent of transcription and translation, (Sadoff, 1972) could also be the consequence of a limited cleavage by the proteases induced at T_0 and therefore just the products to fill the pools. The reversibility of this early period (Sterlinski and

Mandelstam, 1969) and the inability of some investigators to identify separate sporulation stages (stage 0, stage I) during this phase with carefully identified mutants (Mandelstam et al., 1975) suggests again that this interval is not entirely related to sporulation-specific events. Bacilli have evolved control mechanisms which allow the cells to reduce net syntheses at the onset of nutrient deprivation for the viable maintenance of the cell. This characteristic is also seen in E. coli, but unlike the coliform bacteria the bacilli have evolved the mechanism of complete dormancy which maintains the cell in an inactive state until the prevailing conditions are such that the vegetative state is once again favored.

Purpose of Study

The report by Hussey et al., (1971) demonstrating a 90-93% "turn-off" of stable RNA synthesis during sporulation was contradicted by Bonamy et al., (1973). In each instance the investigators studied the incorporation of radioactivity during a twenty minute labeling period, into the assembled 50s and 30s ribosomal subunits. It seemed possible that a more accurate measure of the transcription of the ribosomal RNA cistrons during sporulation would be to observe all the transcripts synthesized and to quantitate the rRNA species from this total population. In this manner one could detect the quantity of rRNA transcribed and not assay just for that portion of the population that was assembled into ribosomes. In these studies the techniques of nucleic acid hybridization and competition were used to identify

The quantity of ribosomal RNA synthesized during the different stages of sporulation.

The major objectives of these studies were as follows, (A) to determine if ribosomal RNA is "turned-off" during sporulation, (B) the level to which it is depressed, (C) the influences of the sporulation medium, stage of sporulation and the cell phenotype on the synthesis of ribosomal RNA and (D) the syntheses of L strand messenger transcripts during sporulation. It is reported that, (1) ribosomal RNA is not "turned-off" during sporulation but rather its synthesis is depressed to levels characteristic of slow growing cells, (2) the quantity of rRNA synthesized during stationary phase is influenced by the nutrient enrichment of the medium, (3) the increase in the quantity and number of different types of L-specific messenger RNAs occurs irrespective of the sporulating nature of the cells, and (4) the depressed levels of stable RNA synthesis observed in stationary phase cells at T_2 can be reversed by the addition of chloromphenicol. This reversal is partial in sp⁺ phenotypes and complete in sp⁻ phenotypes. It is suggested that the initial changes in the quantity of rRNA synthesized during stationary phase are independent of the sporulation phenotype and represent a decrease in the RNA synthetic rate possibly induced by MS or HPN nucleotides. Changes occurring after T_1 , or after the transition period, are specific to sp⁺ cells.

MATERIALS and METHODS

BACTERIAL STRAINS

Bacillus subtilis, strain A26U⁻, a sporulating 168 derivative which requires uracil was used as the reference spore wild-type. All other strains used in these experiments are presented with their characteristics in Table 1.

RADIOISOTOPES

The isotopes listed below were used throughout these experiments:

(¹⁴C)-L-phenylalanine, 260 mc/mmole, Schwarz BioResearch.

(³²P) H₃PO₄, carrier free, Schwarz BioResearch.

(³²P) K₂HPO₄, 800 mc /mmole, New England Nuclear.

(³H) Uridine (5', 6'), 46.2 c /mmole, New England Nuclear.

GROWTH and SPORULATION of BACILLUS SUBTILIS

A variety of media were used in these experiments for vegetative growth and sporulation. The procedures used with each medium differed and therefore each method will be described in detail. In general, cells were inoculated into Penassay (Difco Antibiotic Selection Medium No. 3) and grown until stationary phase. A dilution of this culture 0.2 ml of a 10⁻³ dilution, was inoculated into the selected growth medium for overnight growth. A dilution of the latter culture is used for the experimental growth cultures. All minimal and defined media were supplemented with the bacterial auxotrophic requirements (s) necessary to support growth. Bacterial growth was monitored

turbidimetrically with a Klett-Summerson Colorimeter equipped with a #66 red filter (620-660 nm). At selected periods during growth or differentiation, the cells were harvested by centrifugation, washed with cold TMA buffer three times (0.05M Tris-HCl pH 7.4, 0.001M $MgCl_2$, 0.01M $NaCN_3$) and either quickly frozen or used directly for the extraction of nucleic acids or protein.

Bacterial strains were maintained on Tryptose Blood Agar Base (TBAB, Difco, Appendix) for continuous use or were stored in the lyophilized state and regrown in Penassay when necessary. Below are listed the media and the methods used for the bacterial growth in each case.

Growth of Cells for DNA Preparation

Veal infusion broth (Difco) containing 0.5% yeast extract (Difco) was used for bacterial growth in the preparation of cellular DNA. The cells were harvested at stationary phase, Klett of 250-300, and the DNA was extracted as described. The wet cell mass per 100ml of culture was 0.5gm at a Klett of approximately 300.

Growth of Cells in Spizizen's Minimal Medium

Spizizen's minimal medium (1958, Appendix) does not support bacterial sporulation. The medium was supplemented with 0.5% glucose, 1.0% sodium glutamate and 0.5% vitamin-free casein hydrolysate (cas. amino acids). This medium served as a defined minimal salts for vegetative growth.

Growth and Sporulation in Double Strength Schaeffer Medium

Double strength Schaeffer medium (2xSG) is an undefined nutrient sporulation medium supplemented with 0.1% glucose, (Leighton and Doi, 1971: Appendix). The bacterial cells are partially synchronized and induced to sporulate by the use of a dilution procedure. An overnight culture of cells is inoculated into fresh 2xSG medium at a Klett reading of 25, and permitted to grow until the beginning of stationary phase (Klett 180; see Figure 11) when a sample of these cells is diluted (1:20) into fresh prewarmed (37°) 2xSG medium. The first observable change from the pattern of logarithmic growth in the fresh medium is defined as T_0 and the beginning of stationary phase. $T_1, T_2, \dots, T_n$ are 1, 2 and n hours respectively in stationary phase.

Growth and Sporulation in Sonenshein and Roscoe Sporulation Medium

The Sonenshein and Roscoe medium, 121B, (1969, Appendix) is a richly supplemented defined medium for the sporulation of B. subtilis. An overnight culture of cells is harvested at room temperature, re-suspended in 121B medium at a Klett reading of 25, permitted to reach stationary phase, harvested and then resuspended at a Klett reading of 50 into fresh prewarmed 121B medium. The onset of stationary phase (T_0) is defined as the first observable change in the pattern of logarithmic growth. $T_1, T_2, \dots, T_n$, are as described above (see Figures 5 & 6).

Growth and Sporulation in Sterlini and Mandelstam Sporulation Media

The Sterlini and Mandelstam media, referred to as SM-R (1969, Appendix) as modified by Dworkin et. al. (1972) is a combination of two defined media used in succession for the induction of sporulation. A culture of cells is harvested and resuspended at a Klett reading of 2 to 4, in a nutrient rich medium (defined as SM) containing 0.2% glucose. After four to five generations of active growth in this medium, corresponding to a Klett reading of 60 to 70, the culture is harvested at room temperature and resuspended at the same cell density in prewarmed nutrient poor medium (R) lacking glucose and amino acids. The time of resuspension is defined as T_0 and $T_1, T_2, \dots, T_n$, are the hours after resuspension respectively.

VIABILITY AND HEAT RESISTANCE MEASUREMENTS

The viable number of cells in a population was determined by making a serial dilution of the cell culture in saline (Appendix A) and plating the cells on TBAB agar plates. The number of heat resistant cells in a population was examined by determining the number of viable cells (V) before and after heating (S) a two milliliter sample of the undiluted growth culture, at 80° for ten minutes. The percent sporulation is expressed as $100 \times S/V$.

pH CHANGES IN THE MEDIA

A pH meter (Leeds & Northrup) was used to measure changes in pH of the growth and sporulation media. Cell suspension, 40 to 50

ml., were centrifuged at 12,000 g for five minutes and the pH of the cell-free supernatant was determined.

ESTIMATION of NUCLEIC ACID and PROTEIN CHANGES DURING DIFFERENTIATION

Colorimetric Analyses

1. Nucleic Acid and Protein

Nucleic acids and proteins were isolated from cells using a modification of the Schmidt-Thannhauser procedure (1945). Duplicate 9.5 ml samples of cell suspension were adjusted to 5% with TCA and stored at 4° overnight. The suspension was centrifuged at 5000 g for 5 minutes and the supernatant discarded. The pellet was re-suspended in 0.5 ml of KOH (0.5 N) to hydrolyze the RNA and incubated at 37° overnight. The sample was neutralized with 0.5 ml of HCl (0.5 N), adjusted to 5% with 1.0 ml of 10% TCA and kept over ice for 3 to 5 hours. The DNA and protein were separated from the hydrolyzed RNA by centrifugation at 5000 g for 5 minutes and the supernatant was used to estimate ribose content by the orcinol method (Schneider, 1957) using yeast RNA (Sigma) as the standard.

The pellet containing the DNA and protein was resuspended in 5% perchloric acid, heated at 80° for 20 minutes to hydrolyze the DNA, cooled to room temperature and centrifuged at 5000 g for 5 minutes to pellet the denatured protein. The supernatant served as the source of hydrolyzed DNA and was quantitatively estimated by the Burton diphenylamine method, (Burton, 1968) as

modified by Giles and Meyers (1965). Calf thymus DNA (Sigma) served as the standard for the diphenylamine reaction.

The protein precipitate was dissolved in water and quantitatively estimated by the Lowry method (Lowry et. al , 1951) using bovine serum albumin (Sigma) as the standard.

2. Alkaline Phosphatase

Alkaline phosphatase activity was estimated using the procedure as described by Torriani (1960). Duplicated 5 ml samples were centrifuged at 5000 g for 5 minutes, the supernatants discarded and the cell pellets stored over ice until the growth experiment has been completed. The pellets were resuspended in 2.0 ml of Tris-HCl buffer, pH 8.0 (1.0 M) containing 5 drops of toluene and incubated at 37° for 30 minutes to make the cells permeable to the substrate. One ml of cell suspension was mixed with 1.0 ml of a 0.1% p-nitrophenol phosphate solution (Sigma) and incubated until the color developed. The reaction was stopped by the addition of 1.5 ml of NaOH (2.0 N) and the cell mixture was clarified by the addition of a spatula-full of barium carbonate and centrifugation at 5000 g for 5 minutes. The difference between the absorbances at 410 nm and 660 nm was used as a measure of the substrate hydrolyzed. Nitrophenol (Sigma) was used as the standard. One unit of enzyme activity is defined as that amount of enzyme that will produce 1 nanomole of nitrophenol per minute at 37°.

Radioisotope Analyses

1. Protein

a. Preparation of Radioactive B. subtilis Protein

Radioactively labeled protein was prepared from B. subtilis strain A26u⁻, by a modification of the Schmidt-Thannhauser procedure (1945). B. subtilis cells were grown in 20 ml of Spizizen's minimal medium supplemented with 0.5% glucose and 1.0% sodium glutamate. During vegetative growth 10 μ c of (¹⁴C)L-phenylalanine was added to the culture and the cells were permitted to grow until stationary phase and then harvested. The cell pellet was resuspended in 5% TCA and incubated at 4⁰ for 30 minutes. The suspension was centrifuged at 5000 g for 5 minutes and washed at 50⁰ in 75% ethanol followed by a 75% ethanol:ether (1:1) wash. The resultant pellet was heated for 30 minutes at 100⁰ in a 5% TCA environment and washed in succession with 75% alcohol, pH 6.0 and ether. The precipitate was dried, dissolved in 0.1 M sodium phosphate buffer, pH 7.5 and stored at -20⁰ until used.

Protein purity is taken as the ratio of the absorbances at 280 nm and 260 nm. The experimental 280/260 ratio was 1.362 and indicates greater than 99% purity. The concentration of the protein in the buffer was determined by using a A₂₈₀ for protein of 1.0. The specific activity of the protein was 80 cpm per μ g.

b. Extracellular Protease Activity

A 5 ml volume of growth culture was centrifuged at 12,000 g for 5 minutes to pellet the cells. The supernatant served as the source of the extracellular protease activity. A 0.1-0.2 ml fraction

of this supernatant was incubated with 8-10,000 cpm of radioactive protein in a Tris-magnesium buffer (0.1 M Tris-HCl, pH 7.5, 0.1 mM MgSO_4) at 37° for 15 minutes. The reaction was stopped by the addition of one volume of cold 10% TCA, chilled on ice for 60 minutes and collected on presoaked GF/C filters (Whatman). The filters were washed with 25 ml of cold 5% TCA, 5 ml of cold ethanol, dried, and counted in a toluene based scintillation fluid.

2. Ribonucleic Acid

The rate of incorporation of (^3H)Uridine at various stages of growth and sporulation were measured by two different methods. Each one will be explained below.

a. Rate of RNA Synthesis, Procedure I

Growth and transfer of B. subtilis strain A26u⁻ in SM-R and Spizizen's minimal medium have been described previously. The only modifications are that the phosphate concentrations in both media (R and Spizizen) were lowered to 0.4 mM and no amino acids were added. At the selected periods during growth and/or sporulation, a 10 ml aliquot of cell suspension was removed from a master culture and mixed with both (^3H)Uridine and (^{32}P)phosphate, each at a final concentration of 35.7 $\mu\text{C}/\text{ml}$. Upon mixing the cells with the radioisotopes, i.e. zero time, at 1, 5, 10, 20, 30 and 40 minutes duplicate 0.5 ml samples were removed, mixed with 5 ml of cold TMA buffer, centrifuged immediately and the pellet resuspended in 2 ml of 5% TCA and kept at 4° overnight. The sample was centrifuged at 5000 g for 10 minutes, the supernatant (TCA soluble fraction) was removed and 0.5 ml of this fraction was mixed with 10 ml

of scintillation fluid (Bray, 1960) for counting. The insoluble precipitate was washed with 5 ml of cold 5% TCA, centrifuged at 5000 g for 5 minutes and resuspended in 0.5 ml of KOH (0.5 N) and was kept at 37° overnight. The hydrolysate was neutralized with an equal volume of HCl (0.5 N), adjusted to 5% with TCA, and kept over ice for 5 hours. The suspension was centrifuged at 5000 g for 5 minutes and 0.5 ml of the supernatant (TCA precipitable fraction) was mixed with 10 ml of scintillation fluid as mentioned above. The rate of RNA synthesis and the size of the acid soluble pools were determined from the slopes of the best fit curves as shown in Figure 9.

b. Rate of RNA Synthesis, Procedure II

Growth and transfer experiments have already been described. At selected period of either growth or sporulation 2 ml of cell culture was removed from a master culture, added to a tube containing (³H)Uridine (5µc/ml) and shaken vigorously for 2 minutes at 37°. At the end of the two minute incubation period, duplicate 0.5 ml samples were distributed into 2 ml of cold TMA buffer and the samples were immediately centrifuged to pellet the cells. The supernatant was discarded and 2 ml of cold TCA (5%) were added to the remaining cell pellet, and the suspension was incubated at 4° overnight. The separation and quantitation of the radioactivity in the TCA insoluble and TCA soluble fractions are identical to that described above.

RADIOACTIVE LABELING OF RNA TO BE USED IN HYBRIDIZATION ANALYSES

Radioactively Labeled Stable RNA

Bacillus subtilis strain A26u⁻ was grown in Spizizen's minimal

medium containing (^3H) Uridine. During vegetative growth, the cells were centrifuged, washed and resuspended in pre-warmed media containing unlabeled uridine (100 $\mu\text{g/ml}$) for forty minutes. Actinomycin D (5 $\mu\text{g/ml}$; Schwartz BioResearch) was added after resuspension for a period of ten minutes to exhaust messenger RNA (Levinthal 1962). The cell culture was adjusted to 0.01 M with respect to sodium azide and poured over crushed ice. The cells were harvested, washed three times with TM buffer (0.01 M Tris-HCl, pH 7.4, 0.005 M MgCl_2) and frozen at -70° and kept at -20° until the pellet was used for the extraction of RNA

Radioactively Labeled Sporulation RNA for a Period of 30 Minutes

B. subtilis strains A26u⁻. 0A5NA and SP98-8 were grown in SM medium in the presence of (^3H)Uridine (10-25 $\mu\text{g/ml}$) and unlabeled uridine (15 $\mu\text{g/ml}$). At a Klett of 40-50, excess unlabeled uridine was added to the growing culture (100 $\mu\text{g/ml}$) for a 20 minute chase period, after which the cells were resuspended into the R medium containing 0.4 mM phosphate as described. At the selected intervals in R medium, 200 ml culture samples were removed from a master culture and mixed with (^{32}P)phosphate (33-70 $\mu\text{g/ml}$) for 30 minutes, followed by rapid chilling with an equal volume of frozen medium. The cells were pelleted as described, washed three times with TMA buffer, frozen at -70° and stored at -20° until used for nucleic acid extraction. The RNA preparations are identified by the stage and the time at which the labeling period was terminated.

Radioactively Labeled Vegetative and Sporulation RNAs for a Period of 3 Minutes

1. Vegetative Cellular RNA

a. B. subtilis strain A26u⁻ was grown in Penassay broth until mid-logarithmic growth, at which time the cells were pelleted and resuspended into Spizizen's minimal medium supplemented with 0.5% glucose, and 1.0% glutamate. During vegetative growth in the minimal medium, the cells were mixed with(³H)Uridine (44 μ c /ml) for a period of three minutes. The RNA extracted from these cells was a generous gift of Dr. Yoshiko Setoguchi. The specific activity of this RNA is 55,200 cpm/ μ g.

b. A 200 ml culture of B. subtilis A26u⁻ cells, vegetatively growing in SM medium were mixed with(³H)Uridine (17 μ c /ml) for a period of 8 minutes. The termination of the pulse-period was achieved by the addition of TMA buffer, and the cells were harvested as described. The specific activity of this RNA is 4,630 cpm/ μ g.

2. Sporulation Cellular RNA

One to ten ml of B. subtilis cells were taken from a master culture at the selected period of growth or sporulation in SM-R, 121B or 2xSG media and mixed with(³H)Uridine (200-400 μ c /ml) for a period of three minutes. The termination of the labeling period occurred by the addition of five volumes of cold TMA buffer to the cell culture. This cell sample was then used for the extraction of RNA according to the Summers' procedure (1969) as described below.

PREPARATION OF MESSENGER-FREE STABLE AND RIBOSOMAL RNAs

I. Growth Conditions

Total stable RNA is composed of ribosomal RNA species in addition to the transfer RNA fraction and is referred to as srRNA. Stable RNA was extracted from vegetative and sporulating cells and therefore will be described separately below.

A. Vegetative Stable RNA

B. subtilis cells, A26u⁻, were grown in 500 ml of Spizizen's minimal medium until late logarithmic growth, at which time rifampicin (30 µg/ml, Schwarz BioResearch) was added for a period of 20 minutes. Following the rifampicin chase period the cells were harvested, washed and frozen as described.

B. Sporulation Stable RNA

B. subtilis, strain A26u⁻, was grown in 500 ml of SM and R media for the preparation of stable RNA synthesized during sporulation. At 2.5 and 6.0 hours after resuspension of the cells in R medium, i.e. T_{2.5} and T₆, rifampicin (100 µg/ml) was added for 40 and 100 minutes respectively. The cells were prepared as described. The stable RNA species isolated from these cells at the indicated periods, i.e. T_{2.5} and T₆, are referred to as S_I and S_{II} stable RNAs respectively.

II. Preparation of Ribosomal RNA from Ribosomes

To vegetative cells of B. subtilis A26u⁻, growing in SM medium, rifampicin (30 µg/ml) was added for 20 minutes. The cells were harvested, washed three times with TMA buffer and frozen at -70° for ribosome extraction. The ribosomes were prepared according to the

procedure of Sun et al. (1972). The frozen cell pellet was thawed (37°) and refrozen (-70°) three times, followed by incubation with lysozyme (1.0 mg/ml, salt-free, Worthington Biochemicals) and pancreatic DNase (10-20 ug/ml, Worthington Biochemicals) at 4° for 20 minutes. The mixture was frozen at -70° and ground in a frozen mortar with alumina. The paste was washed from the mortar and the pestle with low magnesium TMN buffer (0.02 M Tris-HCl, pH 7.4, 0.001 M $MgCl_2$, 0.6 mM β -mercaptoethanol, 0.5 M NH_4Cl) and the cell lysate was treated with a series of centrifugations (once at 7000 g, twice at 20,000 g and three times at 30,000 g) to remove the alumina, cell debris and DNA from the supernatant. The supernatant resulting from the last centrifugation (S-30 fraction) was dialyzed at 4° against 2 changes of TMN buffer. A 3 ml sample of the dialyzed S-30 extract was layered on 35 ml of a 1.1 M sucrose-TMN buffer solution. The sample was spun at 40,000 rpm in 40 T_i rotor at 4° for 18 hours, which resulted in the pelleting of ribosomes. The pellet was resuspended in TMA buffer and the RNA extracted with phenol.

EXTRACTION OF NUCLEIC ACIDS

1. Ribonucleic Acid Extraction

Since the procedure and method employed for the extraction of RNA from a cell culture was determined by the cell mass, two procedures are presented below.

a. RNA Extraction from a Large Cell Mass, Procedure I

The RNA was extracted from a cell suspension in which the range of dry cell mass varied between 50-500 mg by a modification of the procedure described by Oishi and Sueoka (1965). A frozen cell pellet was thawed at 37⁰ and refrozen at -70⁰ three times. The thawed pellet was resuspended in 5 ml of TMA buffer, treated with lysozyme (1 to 2 mg/ml) and pancreatic DNase (20 µg/ml) at 4⁰ for 20 to 30 minutes, adjusted to 1% with respect to bentonite (Fraenkel-Conrat et al. 1961; Singer and Fraenkel-Conrat, 1961) and quickly frozen at -70⁰. The frozen suspension was ground in a frozen mortar with two grams of alumina and 5 to 10 grams of glass beads (0.1 mm) until liquification of the frozen suspension was achieved. At that time the suspension was adjusted to 1% with recrystallized sodium dodecyl sulfate and shaken with an equal volume of 90% phenol (redistilled) at 4⁰, followed by centrifugation at 12,000 g for 5 minutes. The aqueous supernatant was removed, mixed with an equal volume of anhydrous ether, shaken at room temperature for 5 minutes, and the mixture was centrifuged for 5 minutes at 12,000 g. The lower aqueous layer was adjusted to 2% with potassium acetate buffer, pH 5.5, and precipitated overnight at -20⁰ with three volumes of 95% ethanol.

The flocculant RNA precipitate was pelleted from the ethanol at -20⁰ and 30,000 g for twenty minutes. The nucleic acid was dissolved in 5 ml of TMA buffer, treated with pancreatic DNase (10 to 20 µg/ml) for 30 minutes at 4⁰, and a second round of deproteinization was repeated beginning with the addition of phenol as described above. The final pellet of RNA was dissolved in TMA buffer (TMA buffer containing 0.005% SDS). The efficiency of this extraction procedure

was 85-90% as determined by the orcinol positive material in the cell suspension.

b. RNA Extraction From A Small Cell Mass, Procedure II

The RNA was extracted from the B. subtilis cells using a modification of the procedure described by Summers (1969). One to ten ml of cell suspension, which ranged between 0.25-10.0 mg dry weight of cells was pelleted and resuspended in 5 ml of protoplasting buffer (0.015 M Tris-HCl, pH 8.0, for 10 minutes at 4⁰, followed by centrifugation at 12,000 g. The pelleted protoplasts were mixed with 200 μ l of diethyl pyrocarbonate (Solymosy et al., 1968), resuspended in 2 ml of lysing buffer (0.01 M Tris-HCl, pH 8.0, 0.01 M NaCl, 0.001 M sodium citrate, and 1.5% SDS) and incubated at 37⁰ for 15 to 20 minutes. At the end of the incubation, the lysate was mixed with 0.5 volume of a saturated solution of NaCl, kept at 4⁰ for 15 minutes and then centrifuged at 12,000 g for 5 minutes. The supernatant, containing the nucleic acids, was precipitated with three volumes of cold 95% ethanol overnight at -20⁰. Additional purification steps are necessary for the removal of DNA and proteins and are performed as described above, beginning with the treatment of the dissolved RNA with DNase. The RNA was dissolved in 5 ml of TMS buffer. The recovery of RNA was 100-120 μ g of RNA per ml of cells at a Klett of 250-300.

2. Deoxyribonucleic Acid Extraction

DNA was extracted by a modification of the Marmur method (1961). The freshly harvested stationary phase cell pellet was washed three

times with a saline-EDTA solution (0.15 M NaCl, 0.1 M EDTA, pH 8.0) and resuspended in the saline-EDTA solution at a cell density of 5 ml solution per gram of cells. Lysozyme (2 mg/ml) was mixed with the cell suspension at 37⁰ with shaking until lysis was complete. The viscous cell lysate was adjusted to 2% with SDS and heated at 60⁰ for ten minutes. The solution was cooled to room temperature, adjusted to 1.0M with sodium perchlorate, shaken vigorously for 30 minutes with an equal volume of a chloroform--isoamyl alcohol solution (24:1 v/v) and centrifuged at 12,000 g for 5 minutes. Two volumes of cold ethanol were added to the aqueous supernatant to precipitate the DNA. The DNA was spooled and dissolved in 0.1 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at 4⁰. The dissolved DNA was mixed with heat treated pancreatic RNase (50 µg/ml, Worthington Biochemicals) and RNase T₁ (50 units/ml, Worthington Biochemicals) for 30 minutes at 37⁰. Self-digested pronase (50 µg/ml, Calbiochem) was then added to the DNA solution and the mixture incubated at 37⁰ an additional 90 to 120 minutes. Further deproteinization of the mixture was achieved by successive mixings of the aqueous DNA solution with equal volumes of 90% phenol (redistilled), anhydrous ether and chloroform-isoamyl alcohol (24:1, v/v) followed by reprecipitation of the DNA in two volumes of cold ethanol. The DNA was dissolved in 0.1 x SSC and a second round of purification was begun with the RNase treatment as described above. After the final precipitation, the DNA was dissolved in 0.1 x SSC and dialyzed overnight at 4⁰ against 1.0 x SSC. The recovery of DNA was 1-2 mg of DNA per gram of wet cell weight.

DETERMINATION OF NUCLEIC ACID CONCENTRATIONS

The UV absorbance of RNA and DNA samples was measured with a Beckman DU model spectrophotometer. The samples were placed in cuvettes and the absorbance at the maximum 260 nm, was measured. A one mg/ml solution of DNA and RNA was determined using an A_{260} of 20 and 24 respectively. The ratio of the absorbancies at 280 nm to that at 260 nm was used to determine the extent of contamination with protein. The expected ratio for pure nucleic acid is 0.5.

METHYLATED ALBUMIN KIESELGHUR (MAK) CHROMATOGRAPHY

1. Preparation of Methylated Albumin

The esterification of bovine serum albumin was according to the procedure of Mandell and Hershey (1960). Bovine serum albumin, "Fraction V" (Schwarz -Mann) was the source of the albumin used. Five grams of the albumin was suspended in 500 ml of absolute (re-distilled) methyl alcohol which contains 4.2 ml of 12 N HCl. Following the 3-5 day incubation in the dark with occasional mixing the mixture was collected by centrifugation and washed two times each with methyl alcohol and anhydrous ether. A powder resulted from the pellet after evaporation. The methylated albumin (MA) was stored in the dry form at -20° , or as a solution in water (1%) at 4° .

2. Preparation of Coated MAK

Twenty grams of kieselghur (Hyflosurpercel, Fisher) were boiled with 100 ml of 0.1 M NaCl in 0.05 M sodium phosphate buffer, pH 6.8 to expel any trapped air (Mandell & Hershey, 1960). The suspension

after cooling was mixed with 5 ml of a 1% MA solution. The mixture was poured onto a column and washed with 250 ml of the buffer followed by 250 ml of a 0.4 M NaCl-0.05 M sodium phosphate buffer, pH 6.8. The kieselghur, now coated with MA was resuspended in 125 ml of 0.4 M NaCl-0.05 M sodium phosphate buffer and used as coated MAK.

3. Preparation of MAK Columns

The columns were packed with the methylated serum albumin absorbed to the kieselghur as described by Mandell and Hershey (1960). The quantity of materials loaded onto the column depended upon the quantity of nucleic acid that was to be fractionated. The following is the recipe for a standard MAK, that is packed into a column having the dimensions 1.9 x 15.5 cm. The bottom and top layers of this four-layered column were a thin layer of cellulose (1.0 g, Whatman Chromedia) in 0.3 M NaCl- sodium phosphate buffer (0.05 M, pH 6.8). The same buffer was used throughout the packing of the column. The second layer contained 8 g of kieselghur boiled in 40 ml of buffer, to which 2 ml of a 1% MA solution was added after cooling. The third layer contained 6 g of kieselghur, also boiled in 40 ml of buffer, but to which was added 10 ml of the coated MAK suspension instead of MA. The two kieselghur layers were packed in 10 ml aliquots, and after addition of the fourth layer, the column was washed with 100 ml of buffer to remove the unbound methylated albumin. The column was always equilibrated with the starting buffer that would be used in the fractionation of the nucleic acids.

4. Fractionation of Nucleic Acids on MAK Columns

a. Deoxyribonucleic Acid Fractionation

DNA was diluted with water to a concentration less than 30 ug/ml and brought to a pH of 12.7 to 12.9 with NaOH (3 N) as monitored by a pH meter. The solution was stirred for a minimum of 10 minutes and then adjusted to pH 6.8 with HCl (3 N). The aqueous solution was dialyzed overnight at 4⁰ against the same sodium phosphate-NaCl buffer that was used at the start of elution from the column, e.g. 0.6 to 0.7 M NaCl-sodium phosphate buffer, pH 6.8. Since hybridization experiments require large excesses of DNA strands, large volumes of DNA (20 to 50 mg) were denatured, dialyzed and stored over chloroform prior to their fractionation on MAK. This procedure removed the variability seen with separate denaturations.

Denatured DNA can be fractionated into complementary strand fractions by the use of an intermittent gradient elution technique first described by Rudner et al. (1968a). DNA, 4-6 mg for a standard MAK, was dialyzed against starting buffer, 0.6 or 0.7 M NaCl-sodium phosphate buffer, pH 6.8, and adsorbed onto a MAK column at a flow rate of 80 ml/hour. The adsorption and the fractionation were followed at 257nm by an ISCO continuous flow monitor. Low molecular weight nucleic acids and native DNA are not absorbed to the column at these salt molarities. A linear gradient of starting buffer and 1.4 to 1.5 M NaCl-sodium phosphate buffer, pH 6.8, (500 ml total) was applied to the column after DNA adsorption. The gradient was discontinued when the first DNA fraction (L-) began to elute (0.75 to 0.8 M NaCl) and

reestablished when the maximum absorbance of that fraction was reached. After all of the first fraction had eluted, the second fraction (H-) began to elute from the column. The eluent was collected in 5 ml fractions. The columns were re-used several times, e.g. 2 to 8 times, after they were initially equilibrated with the starting buffer to be used in the next fractionation.

The complementary DNA strand fractions (L-, H-) eluted from the column were pooled into separate L or H fractions, dialyzed against 0.1 x SSC overnight at 4⁰ and either used directly for hybridization reactions, or concentrated as described below and then used in the hybridization reactions. In some experiments the pooled DNA fractions were concentrated after dialysis by (1) ethanol precipitation, (2) dialysis against dry sucrose, (3) rapid elution from a MAK column by using a high salt buffer, e.g. 2.0 M NaCl, or (4) dialysis against dry polyethylene glycol (Fisher). All these procedures except for the last one, gave similar hybridization efficiencies to that observed with unconcentrated DNA. Polyethylene glycol however, removed all hybridization properties of the DNA, ie. less than 3% of the original hybridization activity remained, and was therefore an unfavorable method of concentration. In the majority of the hybridization experiments, unconcentrated DNA fractions were used.

b. Ribonucleic Acid Fractionation

Fractionation of RNA on MAK columns has already been described by Kano-Sueoka and Spiegelman (1962), Margulies et. al. (1970a,b) and Testa and Rudner (1975). A 2 mg aliquot of the original extracted RNA dissolved in 0.3 M NaCl-sodium phosphate buffer, pH 6.8, at a concentration of 20 to 25 $\mu\text{g/ml}$, was applied to a standard MAK column

which was equilibrated with that saline-buffer solution. When two independently isolated RNA samples were co-chromatographed, they were mixed together and applied to the column in the starting buffer. After adsorption of the RNA sample to the column, an equal volume of starting buffer was applied as a wash, followed by the elution of the RNA by a linear salt gradient e.g. 0.3 M to 1.2 - 1.4 M NaCl in sodium phosphate buffer pH 6.8 (total volume 500 to 600 ml). The RNA species were eluted from the column according to size, the smallest species eluting first. Greater resolution between the 16S and 23S ribosomal RNA species was obtained by interrupting the gradient as the 16S fraction began to elute and to reestablishing the gradient after the maximum absorbance of the 16S peak was reached (Margulies et al. 1970a).

When ^{32}P -labeled samples were eluted from a MAK column the RNA was freed of extraneous ^{32}P -label in protein and polysaccharides which prevent the accurate determination of specific activities and the quantitative assay of DNA-RNA hybrids on filters. This procedure involves the adsorption of the RNA to a MAK column, extensive washing of the column with the low salt buffer (0.3 M NaCl), and rapid elution of the "clean" RNA from the column by 2.0 M NaCl in sodium phosphate buffer. The eluted RNA, once dialyzed against water and 0.1 x SSC, can be precipitated with two volumes of ethanol as described. The precipitated RNA is dissolved in TMS buffer and used directly for hybridization experiments.

HYBRIDIZATION OF MAK SEPARATED L AND H DNA STRANDS WITH RADIOACTIVE RNAs

The liquid hybridization technique of Nygaard and Hall (1963) as modified by Kennell and Kotulas (1968) was used to hybridize RNA to MAK L and H DNA strands as described by Margulies et al. (1968a,b). DNA fractions free of their complementary strands were annealed in solution to radioactively labeled RNA samples in the presence or absence of unlabeled competitor RNA. The incubation of the nucleic acids in 6 x SSC at 67° for 18 hours, took place in screw cap test-tubes. At the end of the incubation the reaction mixture was diluted with 10 to 15 ml of cold 2 x SSC and collected on prewashed nitrocellulose filters (24 mm, B-6, Schleicher and Schuell). The filters were washed with 100 ml of 2 x SSC and incubated with 5 ml of 2 x SSC containing heat treated pancreatic RNase (20 µg/ml) and RNase T₁ (20 units/ml) for 60 to 90 minutes as outlined by Gillespie and Spiegelman (1965). The filters were then washed on each side with 50 ml of 2 x SSC, dried and counted in a toluene based scintillation fluid. Reaction volumes for each hybridization experiment were different and therefore these volumes are presented in the respective legends to the tables and figures for the hybridization results.

DETERMINATION OF RADIOACTIVITY

A Packard Tricarb Liquid Scintillation Counter was used in these experiments. The settings used for counting were 70-1000 with a 50% amplification and 70-1000 with a 2.4% amplification for tritium and (³²P)radioisotopes respectively. When both(³H)and(³²P)isotopes were counted simultaneously in the same vial, the settings were 70-750, 50% amplification and 130-850, 1.1% amplification respectively.

The spill of the (^{32}P) into the (^3H) channel was determined to vary between 1.34% and 5.0% and was subtracted from the tritium counts. (^{14}C) labeled protein was counted using a setting of 70-1000 with an amplification of 15%.

Aqueous samples were counted in the Bray scintillation fluid (Bray, 1960) which was prepared as follows: solution A contained 120 g of naphthalene (Fisher), 4 g of PPO (New England Nuclear), 200 ml methanol (Fisher) and 40 ml of ethylene glycol (Fisher) added to 1.0 liter of p-dioxane (Fisher). Solution B contained 4 g of POPOP (New England Nuclear) dissolved in 1.0 liter of p-dioxane. The two solutions were mixed. The ratio of the aqueous sample to the amount of scintillation fluid used varied from 1:3 to 1:10 between experiments but was constant in an individual experiment.

A toluene based scintillation fluid composed of 6g of PPO and 0.5 g POPOP per liter of toluene (Fisher) was used for counting all samples bound to filters. The counting efficiency of (^3H)Uridine was 32.4%.

RESULTS AND DISCUSSION

Sporulation is the sequential adjustment made by bacilli in response to environmental depletion of carbon, phosphorous and/or nitrogen sources (Shaeffer, 1965). The dormant spore remains prepared to resume active growth should conditions become favorable. As a spore, the bacillus is resistant to the environmental forces that would have caused the destruction of the vegetative cell. As it has been shown (Young and Wilson, 1972) the events leading to the resultant spore appear in sequentially ordered distinct periods. These developmental changes as they have been identified biochemically and morphologically are separated into distinct stages for easy identification (see Figure 1). The sporulation sequence is ordered and invariable in the bacilli regardless of the sporulation medium used. The end of vegetative growth, T_0 , believed to be synonymous with the onset of sporulation, is the most difficult time to define in a sporulation medium. In order to establish the various parameters responsible for the induction of sporulation, it was first necessary to characterize and define the conditions of growth and sporulation. The Sterlini and Mandelstam sporulation media (1969; SM-R) was used in the majority of these studies because of the following: (1) T_0 is well defined and reproducible, (2) B. subtilis sp⁺ strains were

found to sporulate with high efficiency and sp⁻ strains could not, and (3) the spore-specific stages were reproducible in this defined medium. T_0 in the other two sporulation media used, the Sonenshein and Roscoe medium (1969; 121B) and the modified Shaeffer medium (Leighton and Doi, 1971; 2xSG), was more difficult to define.

CHARACTERIZATION OF GROWTH AND SPORULATION IN BACILLUS SUBTILIS

These sporulation studies were performed with the Bacillus subtilis strains listed in Table 1, in a variety of growth and sporulation media (Appendix A) to identify those macromolecular changes which were specific to the developmental sequence. The parameters studied were compared to similar events that occurred in vegetative cells and in non-sporulating cells. B. subtilis strain A26u⁻ was used as the reference sporulation wild-type (sp⁺) throughout these studies although other sp⁺ strains were also used. The asporogenous (sp⁻) cells chosen for this study were all blocked genetically at or before stage 0 of sporulation which prevented the cells from entering the typical sequence of events leading to spore formation as outlined in Figure 1. These Spo0 mutants were useful in determining the changes which occurred during the onset of sporulation. Certain properties of these sp⁺ and sp⁻ cells are presented in tables 1, 2 and 3.

The transition period, as discussed in the introduction and as defined in these studies, is the period in stationary phase from T_0 to T_1 . It was during this period that certain characteristics were shown to be common to the stationary phase B. subtilis cells

studied irrespective of the spore genotype, i.e. depressed rates of RNA synthesis, lower levels of rRNA transcription and increased quantities of messenger RNA transcription. This transition period is described here since references are made to it throughout this discussion of the results.

Nucleic Acid and Protein Metabolism in SM-R Media

The growth and sporulation related parameters measured in these studies, i.e. net macromolecular syntheses, cell viability, heat resistance, pH fluctuations and alkaline phosphatase activity, as they appeared in A26u⁻ cells sporulating in SM-R media (Sterlini and Mandelstam, 1969) are presented in Figure 3. This media consists of two separate parts, a nutrient rich growth medium (SM) containing high concentration of amino acids, glucose and phosphate, and a nutrient poor sporulation medium (R) lacking amino acids, glucose and containing low levels of phosphate but a very high magnesium content. Glucose (0.2%), not present in the original recipe by Sterlini and Mandelstam, was added to the SM medium in order to increase the growth rate of the vegetative cells. Early reports by Blassa (1963) and Spotts and Szulmajster (1962) demonstrated that protein and nucleic acid accumulations stopped at the end of vegetative growth. This shutdown of net macromolecular syntheses is believed to be a result of the events leading to the initiation of sporulation. Panel B of Figure 3 shows

the rapid halt in RNA and protein syntheses, reminiscent of the shift-down behavior displayed by cells during a carbon source shift-down, which has been shown to result in the immediate cessation of RNA and protein accumulation (Kurland and Maaløe, 1962; Lazzarini and Winslow, 1970). DNA synthesis, which is also depressed, comes to a gradual halt after T_2 . Although the cellular content of RNA during the first five hours of stationary phase remained constant, there were changes in the synthetic rates and in the types of RNA species made during this developing period, and these will be described later.

Alkaline Phosphatase Activity in SM-R Media

Glenn and Mandelstam (1971) have shown that the alkaline phosphatase enzyme isolated from vegetative and sporulating cells is virtually identical. The control of its expression, however, appears to be regulated differently in growing and sporulating cells, since alkaline phosphatase-negative cells unable to synthesize the enzyme during vegetative growth, will synthesize the enzyme during sporulation. The enzyme is induced even in the presence of sufficient phosphate in the sporulation medium (Grant, 1974). Alkaline phosphatase activity was selected as an early marker of sporulation-specific biochemical events since its induction and accumulation during sporulation are under the control of the differentiating system and not solely due

to the effect of phosphate starvation (Grant, 1974). This activity, as reported by others, appears by T_2 and therefore corresponds to an early sporulation activity and not a change occurring during the transition period, i.e. T_0 - T_1 . The appearance of alkaline phosphatase activity as it has been reported, in the resuspension (R) medium is presented in panel C of Figure 3 (Sterlini and Mandelstam, 1969; Testa and Rudner, 1975b). The enzyme activity consistently appeared by T_2 and reached a maximum activity by T_6 in the R medium.

Heat Resistance (S/V) in SM-R Media

Heat resistance is a physical measurement of the ability of a cell population to withstand high temperatures for extended periods of time. Vegetative cells did not survive this treatment (80° for 10 minutes) that leaves spores unharmed. Heat resistance as shown in Figure 3 and reported by Sterlini and Mandelstam (1969) appeared routinely between T_6 and T_8 , and reached a maximum by T_{10} . The heat resistant property is taken to represent the final stage of sporulation in all the sporulation media used in these studies.

pH Changes During Sporulation in SM-R Media

Earlier reports (Szulmajster and Hanson, 1965; Kawamura and Ito, 1975) have shown that during vegetative growth in a nutrient sporulation medium, there is a rapid decrease in the pH of the medium due to the formation of acids, i.e. pyruvic and acetic, during glycolysis.

Upon entering stationary phase, the pH of the external medium increased steadily, e.g. pH 8.4, as the developing bacteria utilized the excreted acids as substrates for the citric acid cycle, the enzymes of which are induced between T_2 and T_3 of sporulation (Szulmajster, 1975). These pH changes, indicative of glucose utilization (SM) medium and the induction of the citric acid cycle enzymes (R medium) are presented in panel A of Figure 3. The initial low pH in the R medium (pH 6.5) is probably the result of the carry-over of some acids from the previous culture medium (SM). The increase in alkalinity, above pH 7.0 was not observed during stationary phase where sporulation is not induced, e.g. SM medium. Therefore it appears that the increased alkalinity of the culture medium is directly related to the process(es) of sporulation.

Asporogenous Cells in SM-R Media

The behavior of the asporogenous cells in SM-R media is shown in Table 2. This table demonstrates that greater than 95% of the sp^+ phenotypes and less than 0.1% of the asporogenous sp^- bacterial strains formed heat resistant spore in the SM-R media. In addition, the characteristic maximum accumulation of alkaline phosphatase activity between T_2 and T_6 of stationary phase in the R medium, was not found in the non-sporulating cells. Early blocked asporogenous mutants of B. subtilis were observed to decrease in viability between T_4 and T_6 during stationary phase in the R medium. This reduction in the viability was presumably due to the inability of these cells to maintain the vegetative state for extended periods during stationary phase or

to perform sporulation functions. Growth analyses of a spo0 mutant SP98-8, in the SM-R media is presented in Figure 4 as a comparison to the observed metabolism of a sporulating cell in the same media (see Figure 3). Although the net accumulations of nucleic acids behaved in a similar manner to that of A26u⁻ sp⁺ cells in the same media, there was no increase in the alkaline phosphatase activity or heat resistance. The high basal levels of alkaline phosphatase in this mutant was not typical of the other spo0 mutants studied. These two parameters were also studied in other spo0 mutant cells (see Tables 2 and 3). The results presented demonstrate that the halt in RNA accumulation during the stationary phase in Bacillus subtilis sp⁺ and sp⁻ cells is not dependent upon the phenotype of the cells but rather seems to be the response of all cells to nutrient depletion and a naturally occurring shift-down in the medium.

Sporulation and Stationary Phase in the Sonenshein and Roscoe

Sporulation Medium (121B)

The medium, 121B, consists of salts supplemented with glucose, amino acids and low levels of magnesium ions. B. subtilis strain NCTC 3610 has the capacity to sporulate in this amino acid sporulation medium, 121B, as first reported by Sonenshein and Roscoe (1969). The characteristic behavior of this strain is presented in Figure 5. The appearances of alkaline phosphatase activity and heat resistance and the decreased accumulation of nucleic acids is typical of the sporulation scheme as presented in the SM-R media and as observed in the double strength nutrient Schaeffer sporulation

medium (2xSG). B. subtilis strain 3610, however, had the ability to sporulate in all three sporulation media used, whereas the other B. subtilis sp^+ derivatives of 168 and W23 died in the 121B medium. Strain A26u⁻, a sp^+ genotype, when grown in 121B medium did not sporulate. This was in strong contrast to the behavior of strain A26u⁻ in the SM-R media (see Figure 3). Figure 6 shows the behavior of strain A26u⁻ in 121B medium. The accumulation of RNA and DNA during the stationary phase behave in a similar manner to that observed for the same strain during sporulation in SM-R media. On the other hand, there was no characteristic accumulation of alkaline phosphatase or heat resistance in this strain or in the other strains studied (see Table 3). In Table 3 the same sporulation parameters are presented as listed in the previous Table 2. B. subtilis sp^+ cells in the 121B medium routinely showed no increase in either of the two sporulation-specific events assayed, i.e. alkaline phosphatase activity and heat resistance. There was a residual fraction of heat resistant spores formed by the sp^+ cells, which never exceeded 11% of the population present at T_2 (the corrected S/V), was still much greater than that observed for the sp^- cells.

Extracellular Serine Protease Activity in 121B Medium

Since the production of sporulation-specific activities are blocked in the sp^+ strains during stationary phase in 121B medium (see Table 3 and Figure 6) it was of interest to more carefully define the point at which the cells were prevented from entering

the sporulation scheme of events. B. subtilis strain A26u⁻ was compared to strain 3610 with respect to its ability to induce the production of extracellular serine protease activity, which is associated with the earliest stage of sporulation (Leighton et al., 1973; Dancer and Mandelstam, 1975a,b). Figure 7 shows the results of such a comparison. Strain 3610, which could sporulate in 121B medium, showed a large increase in the activity associated with the serine protease by T₀ (see panel A of Figure 7) which was inhibited by the addition of a serine protease inhibitor, i.e. PMSF, phenylmethanesulfonyl fluoride (Gold and Fahrney, 1964). Strain A26u⁻ however, displayed no such increase characteristic of induction and the extracellular and intracellular proteolytic activities were not inhibited by the addition of PMSF. The absence of proteolytic activity associated with the serine protease does not distinguish between the total absence of the enzyme(s) and the production of inactive enzyme molecules.

The importance of serine protease during stage 0 of sporulation has been well established. Leighton et al. (1973) have characterized a temperature-sensitive serine protease mutant. This mutant at the nonpermissive temperature is prevented from sporulating and does not produce an active serine protease whether assayed in vivo or in vitro. Strain A26u⁻ is blocked at or before stage 0 of sporulation as a result of some compound in 121B medium. This phenomenon, is demonstrated in Table 3 and Figures 6 and 7 as the inability of sp⁺ cells to induce sporulation-specific functions and is referred to as a medium conditional phenocopy, i.e. SpoM_c.

SpoM_C will hereafter be referred to in these studies, when describing medium induced asporogenous cell phenocopies. Pun et al. (1975) have identified the reverse situation in a rifampicin-resistant sp⁻ genotype of Bacillus subtilis. A cell culture incapable of sporulating in SM-R media could be induced to sporulate by the addition of amino acids to the resuspension (R) medium. These sp⁻ strains are probably different from the sp⁺ strains used in this study, although they are both suggestive of changes in the RNA polymerase apparatus. Although these sp⁺ strains can not sporulate in 1218 medium they can be used for the characterization of the transition period, in conjunction with the sp⁻ strains.

RELATIVE RATES OF RNA SYNTHESIS DURING NUTRIENT DEPLETED GROWTH CONDITIONS

Reports from different laboratories have demonstrated a template specificity change in the Bacillus subtilis RNA polymerase occurring early during sporulation, i.e. the first two hours. This change was observed and in vitro as decreases in the synthesis of ribosomal RNA (Hussey et al., 1971, 1972) and the production of bacteriophage ϕ e (Sonenshein and Roscoe, 1969; Losick and Sonenshein, 1969). Sumida-Yasumoto and Doi (1974) in addition have shown that during this period of the template specificity change, there is a rapid decrease in the rate of RNA synthesis. Figures 3-6 have demonstrated that the halt in RNA accumulation, coinciding with T_0 of stationary phase, is not specific to sporulation since it occurs in sp⁺, sp⁻ and SpoM_C cells in the different media used. If this abrupt change in the net increase in RNA is not specific to sporulation, it may be due to a response of the cells to a naturally occurring depletion of some essential nutrient, i.e. carbon, nitrogen, phosphorous, in the growth medium. If this assumption is correct, the regulation of RNA synthesis during this period may be similar to that observed in cells shifted from a nutrient rich to a nutrient poor medium. Maaløe and Kjeldgaard (1966) have reported that net RNA and protein synthesis come to an almost complete stop when cells grown in a broth medium are transferred to a glucose minimal salts medium. These cells show no accumulation of macromolecules until they have adjusted their metabolism to the new growth medium. Similar results

were observed in minimal salts media by shifting cells grown in glucose to a medium lacking glucose and containing acetate as the carbon source (Khan and Yamazaki, 1974) or by the addition of a competitive inhibitor of glucose, i.e. alpha methyl glucoside (Hansen et al., 1975).

To further establish whether the decreased synthetic rates of sporulating cells were specific to the process of sporulation or just the consequence of adjustment to slower growth and to nutritional shift-down during stationary phase, a series of experiments were designed to determine the relative rates of RNA synthesis and the sizes of the acid soluble pools.

Glucose Shift-Down

An experiment was designed to induce a carbon shift-down condition in B. subtilis A26u⁻. The cells were grown in a minimal salts medium (Spizizen, 1958) supplemented with 1.0% glutamate, 0.5% casein hydrolysate and 0.5% glucose (Medium I). During vegetative growth in Medium I, a sample of cells at a Klett of 100, was centrifuged and resuspended in an identical prewarmed (37°) medium lacking glucose (Medium II). At selected intervals in both media I and II, samples were withdrawn and mixed with (3H) Uridine for a two minute pulse-label as described in the Material and Methods, and the TCA soluble and insoluble radioactivity determined. The results are presented in Figure 8. Panel A demonstrates the uptake of the label by the cells during growth and stationary phase in Medium I. Throughout the growth

phase there was an increase in the quantities of radioactivity present as TCA precipitable RNA and in the acid soluble pools due to the increases in cell mass. At the onset of stationary phase, i.e. T_0 , the quantity of RNA synthesized decreased steadily at a rate of 10% per hour. Similar quantities of TCA soluble radioactivity were observed for T_0 and stationary phase (see Figure 8A), demonstrating only a small decrease in the uptake of radioactivity occurred during the latter phase. In that minimal medium, as noted earlier, sporulation does not occur, e.g. S/V was less than 5%. Panel B shows the growth and uptake of (^3H)Uridine by the cells that were removed from Medium I at midlogarithmic growth, i.e. R_0 (see Figure 8A), and resuspended in Medium II at the same cell density, in the presence and absence of chloramphenicol. A discussion of the effects of chloramphenicol (CM) will be presented later. The cells in Medium II do not increase in optical density during their adjustment to a new carbon source. After the adjustment period or transition phase, the cell mass of the culture is seen to increase at 20% of the preshift rate. Similarly, the rate of RNA synthesis during the transition phase is only 3-5% of the preshift value observed at R_0 , which could not be accounted for by the observed changes in the soluble pools. After the cells in Medium II adjusted to using the new carbon source, the rates of RNA synthesis were observed to increase to 40-50% of the original preshift rate. In conclusion, a carbon source shift-down causes a period of decreased RNA synthesis until the cell population adjusts to a new carbon source, which is observed as an increase in

the cell mass of the culture and an increase in the levels of RNA synthesis after this transition period, in other words, as growth resumes.

Sporulation

The Sterlini and Mandelstam growth medium (SM; see Appendix A) is a medium supplemented with 0.2% glucose, 10 mM phosphate and a very high content of amino acids (greater than 0.8%, final concentration). The cells are induced to sporulate by their transfer to a medium (R) lacking both glucose and amino acids, and supplemented with only 0.5 mM phosphate but relatively high levels of magnesium ions, 40 mM. In essence, this transfer induces glucose, amino acid and phosphate shift-downs in the cells. The effects of a glucose shift-down on the rates of RNA synthesis have been described previously (see Figure 8).

The rates of RNA synthesis during sporulation in SM-R media were determined for a comparison to the synthetic rates observed in slowly growing cells. Two sources of radioactivity, (^3H)Uridine and (^{32}P)phosphate, were used throughout these studies. It was therefore of interest to examine if any observable differences existed in the uptake of the radioisotopes into the acid soluble pools or in the rates of RNA synthesis. Cells of A26u⁻ were doubly labeled with (^3H)Uridine and (^{32}P)phosphate throughout vegetative growth and sporulation in SM-R media. A ten ml sample of the cell culture was mixed with both radioisotopes (each at a final concentration of 35.7 μC /ml), and samples of the labeled culture

Were removed at different time intervals. The labeling of the cell culture was stopped by the addition of ice cold TMA buffer, followed by the centrifugation of the cells. The washed cell pellet was resuspended in cold TCA (5%) and the soluble and insoluble radioactivity, as a function of time, determined (see Materials and Methods, Procedure I). The results of one such experiment performed at sporulation stage T_2 in R medium is presented in Figure 9. Similar experiments were performed at midlogarithmic growth and sporulation stages T_0 - T_6 . In general, the two to four-fold greater quantity of radioactive counts (cpm) consistently observed in the (^{32}P) acid soluble pools, when compared with the (^3H)Uridine soluble pools could result from the (1) higher counting efficiency of the (^{32}P)-isotope, (2) use of the (^{32}P)phosphate to phosphorylate each of the four ribonucleosides, and/or (3) catabolic breakdown of the (^3H)Uridine into carbon and nitrogen sources. Equilibration of the soluble cell pools with the externally added radioisotopes, (^{32}P) and (^3H)Uridine, was observed immediately within the first minute and remained constant for the next forty minutes (see Figure 9A). The level used as a comparison between the different states of growth and sporulation was that quantity of radioactivity present in the soluble cell pools after 20 minutes of exposure to the radioactivity. The rate of RNA synthesis at T_2 , and the other stages measured, were determined from the slopes of the plots: specific activity of the RNA (cpm/ μg RNA) versus time (see Figure 9B).

A composite figure of the relative rates of RNA accumulation and soluble pool changes for the different stages of sporulation is presented in Figure 10. This figure shows that identical labeling patterns are observed for each radioisotope, (^3H)Uridine and (^{32}P)phosphate, when they are normalized to the rates of RNA synthesis and the soluble pool sizes present during vegetative growth. Although the absolute incorporation into RNA or the soluble pools may vary with the isotope used, the changes observed during sporulation were identical. Similar to the carbon source shift-down is the decreased rates of RNA synthesis observed during the first hour after the cells were resuspended (compare Figure 10 to Figure 8B). This sporulation transition phase however, which lasts the same amount of time as seen in the shift-down medium, does not return to levels of RNA synthesis observed during growth in a medium lacking glucose, i.e. 50% of the preshift R_0 value (see Figure 8B). During sporulation, after the transition phase, RNA synthesis is maintained at a rate 10% of that observed in vegetative growth. This low level is not greatly influenced by the size of the soluble nucleotide pools, which remain constant during the first two hours of sporulation and decrease slowly at a rate of 10% per hour afterwards. These results demonstrate that cells in the SM-R sporulation media exhibit the following: (1) a rapid decrease in the rates of RNA synthesis during T_0 - T_1 in R medium, due to a similar carbon shift-down phenomenon described earlier, and (2) the rate of RNA synthesis after this transition period is maintained throughout the sporulation process at its low levels,

10% of the vegetative rate.

In order to substantiate these observations, two other sporulation media, 2xSG and 121B, that do not require a shift-down transfer were examined with respect to the rates of RNA synthesis. Strain A26u⁻ was grown in the nutrient sporulation medium, 2xSG and samples of the cell culture were mixed with (³H) Uridine for two minutes and the radioactivity determined as described previously. The results presented in Figure 11 demonstrate a rapid decrease in the rate of RNA synthesis by $T_{0.5}$, to 27% of the T_0 rate. This transition phase rate decrease is not as low as that observed in SM-R media during the early transition period of sporulation (see Figure 10). In this nutrient sporulation medium, the initial rapid decrease in the synthetic rate is followed by a slower decline, until T_2 when the rate is maintained at only 5-10% of that observed during vegetative growth. An identical pattern for a decrease in the rates of RNA synthesis in 2xSG medium has been reported by Sumida-Yasumoto and Doi (1974). The acid soluble pools however, rapidly decreased by $T_{0.5}$ and were maintained at 50% of the level found at the beginning of stationary phase. This is in contrast to SM-R media, in which there is no change in the soluble pools until T_2 .

Bacillus subtilis strain 3610 sporulating in 121B medium was used as another sporulation system in which the rates of RNA synthesis could be studied. Figure 12 demonstrates a very similar RNA synthetic pattern to strain A26u⁻ in 2xSG medium (see Figure 11). Again the soluble radioactive pools decrease to half the level found at the onset

of stationary phase. It is interesting to note that the decreases in the rate of RNA synthesis and the amount of isotope taken into the acid soluble pools of the cell, was established between T_1 and T_2 . This pattern is very similar to the patterns observed in the other two media studied. In addition, cells in all three media induce the production of alkaline phosphatase at T_2 (see Figures 3 and 5 for SM-R and 121B media respectively; 2xSG not presented) suggesting that the cells induce sporulation events at similar times although the sporulation media differ. These results indicate that the initial change in the rate of RNA synthesis during the transition period occurring between T_0 - T_1 , may be due to the depletion of some nutrient (glucose, phosphate, nitrogen) from the growth medium. The period from T_1 - T_2 may be the stage when the lower RNA synthetic rate is established by a mechanism other than that observed during a shift-down, e.g. loss of vegetative sigma subunit activity as reported by Segall et al. (1974). The establishment of the slower growth rate, decreased nucleotide pools, decreased DNA synthesis, etc. could initiate the observed sporulation-specific processes like alkaline phosphatase production.

Asporogenous Cells: Phenocopies ($SpoM_c$) and SP^- Genotypes

As shown earlier (see Figures 6 and 7) some Bacillus subtilis strains, A26u⁻, W23 and BD29, when grown in 121B medium are unable to sporulate, although they are genotypically sp⁺ strains (see Tables 2 and 3). Strain A26u⁻ was shown to mimic a block before the

production of an active serine protease and therefore resembled a spo0_a genotype. It was of interest to check to rates of RNA synthesis in these SpoM_C strains during growth and stationary phase in 121B medium. Similar two minute pulse-labeling experiments were performed with these cells as described in Figure 8. The results of these experiments are presented in Figure 13. The SpoM_C phenotype of strains BD29 and W23 demonstrate RNA rate transitions very similar to those observed during sporulation in 2xSG and 121B media (compare Figures 11 and 12 with 13 B and C). Both these strains BD29 and W23, can not produce alkaline phosphatase and are therefore metabolically blocked before T_2 . They do show a shift in the rate of RNA synthesis between T_0 and T_1 and appear to mimic a metabolic block before or during the initiation of sporulation-specific events. Strain A26u⁻ on the other hand, mimics a much earlier block. There is only a small decrease in the rate of RNA synthesis and no decrease in the size of the nucleotide pools for the first five hours in the stationary phase (see Figure 13A). In fact, the pattern of RNA synthesis and pool sizes behave very similarly to those observed for A26u⁻ in another medium which does not support sporulation, ie. Spizizen minimal salts medium (see Figure 8).

The data presented in Figures 8-14 are presented in a summary form in Table 4. In each example shown in this table, there is a rapid decrease in the rate of RNA synthesis evident by T_1 , except for A26u⁻ grown in media which do not support its sporulation, ie. 121B and Spizizen minimal salts medium.

Strain A26u⁻ has been shown to be metabolically blocked from entering the earliest stage of the sporulation sequence when grown in 121B medium, and it was of interest to compare its rates of RNA synthesis during stationary phase in that medium with known genetically blocked spo0_a mutant and rfm3Y, a spo0 mutant, are blocked early in the sporulation sequence of events (see Figure 1 and Table 1). The mutant cells were grown and pulse-labeled with (³H)Uridine in the Sonenshein and Roscoe medium (121B) under identical conditions used for the study of the SpoM_C phenocopies. Under these conditions, less than $5 \times 10^{-2}\%$ of the mutant cell population sporulated (see Table 3). The results of these experiments are presented in Figure 14. In both mutant cell lines, SP98-8 and rfm3Y, a RNA synthesis rate transition was observed to occur by T_{0.5}. This low synthetic rate, which was maintained during the stationary phase, was very similar to the rates observed in sporulating cells (sp⁺; see Figures 11 and 12) and nonsporulating cells (SpoM_C; see Figures 13 B and C).

The similarity between sp⁺, sp⁻ and SpoM_C phenotypes, with respect to the rate transition observed in the first hour of the stationary phase, makes it doubtful that this decreased synthetic activity is specific to the sporulation sequence. On the other hand, strain A26u⁻ in 121B medium (see Figure 13 A) consistently maintained high rates of RNA synthesis for three to six hours beyond T₀. This high rate of RNA synthesis during stationary phase was not decreased significantly by lowering the magnesium ion concentration of 121B medium

(see Table 4). The rapid decrease in the rates of RNA synthesis coinciding with T_0 may reflect a change in the RNA polymerase which occurs as a response of the cell culture to a change in the medium e.g. glucose deprivation, and occurs in sp+, sp- and SpoM_C cell phenotypes. B. subtilis strain A26u⁻ grown in 121B medium is prevented from undergoing this rate transition and is blocked before the earliest reported event in the sporulation sequence.

In conclusion, the abrupt change in the rate of RNA synthesis during the first hour of sporulation is a pre-or co-requisite for the initiation of sporulation specific events, since A26u⁻ cells stopped from this rate transition are also prevented from sporulation. The period between T_0 and T_1 when the rate of RNA synthesis is observed to decrease in stationary phase cells, is referred to as the transition period.

CHROMATOGRAPHIC PROPERTIES OF VEGETATIVE AND SPORULATION

RNA TRANSCRIPTS

A study of the overall rates of RNA synthesis during growth and sporulation can only identify periods of gross metabolic changes occurring in a cell population. In order to study the synthesis of stable and unstable RNA species, more sensitive analytical methods must be employed. The bulk RNA content in a bacterial cell is stable RNA (srRNA) composed of ribosomal RNA (rRNA) and transfer or soluble RNA (t or s RNA) species. Greater than 95% of the isolated RNA within a cell is stable RNA (Midgley, 1969; Norris and Koch, 1972) and the remaining species are a mixture of RNAs called messenger RNA (mRNA) and precursors for rRNA and tRNA. Stable RNA species can be preferentially labeled with radioactivity because they are not turned-over in vivo as rapidly as the messenger RNA fraction (Leighton and Doi, 1971). The addition of labeled nucleosides followed by the addition of unlabeled nucleosides will cause the radioactivity to remain longer in the more stable species i.e. tRNA and rRNA. In this way, greater than 98% of all radioactivity can be found to reside in the srRNA fraction. To analyze the unstable RNA fraction which appears as part of the total population of RNAs, cells are exposed to radioisotope for short periods of time compared to the doubling time of the cell population. The shorter the period of time of exposure to the isotope, the greater the proportion of label that will reside in the mRNA. This proportion is dependent upon the growth rate of the cells at the time

of labeling (Midgley, 1969). Further identification of each RNA class, e.g. srRNA and mRNA, can be made by the use of nucleic acid hybridization and competition techniques (Gillespie and Spiegelman, 1965; Kennel and Kotoulas, 1968; Avery and Midgley, 1969) in conjunction with the use of two different radioisotope labels. In the studies presented here, both (^3H -Uridine and (^{32}P)phosphate were used together to label different RNA populations. The (^3H)isotope was used to label only vegetative stable RNA and the (^{32}P)isotope was used to label those RNA species synthesized during sporulation. In this fashion, the presence of two isotopes permitted more extensive analyses of the changes occurring in the transcripts synthesized and turned-over during sporulation.

B. subtilis A26u⁻ was grown in SM medium in the presence of (^3H)Uridine for four generations (90 minutes) and then transferred to the resuspension medium (R) containing unlabeled uridine for a series of 30 minute labelings with (^{32}P)phosphate. A sample of the extracted RNA was dissolved in 0.3M NaCl-0.05M sodium phosphate buffer, pH 6.8 and applied to a MAK column. The elution profile of the RNA isolated from sporulating cells at T_2 is presented in Figure 15. The profiles observed are those for (^3H)labeled vegetative srRNA and a mixture of srRNA and mRNA labeled with (^{32}P) during sporulation in R medium. The ultraviolet absorbance profiles of the 4+5S, 16S and 23S RNA species were used as the internal standards for the column elution profile. It is seen from this figure that the tritium isotope resides in the srRNA species and exhibits a 2:1 ratio in the high molecular weight region expected for pure 23S:16S species. On the other hand, the high molecular weight material labeled with

(^{32}P) does not display this ratio, suggesting that mRNA may be present, or that some of the 23S species may have degraded into the 16S region of the column.

Low molecular weight material, i.e., degraded RNA, DNA, teichoic acids and other non-nucleic acid materials were excluded essentially with the application of the RNA sample. This early eluting region is referred to as peak I. During sporulation there was an increase in the quantity of tritium label eluted in this low molecular weight region, implying a turnover of the pre-existing (^3H) labeled vegetative srRNA during sporulation. Figure 16 shows the appearance of peak I with the stage of sporulation. Each point is the average of two or more independent radioactive labeling experiments. The slope of this curve was used to calculate the turnover rate and half-life of the vegetative srRNA species during sporulation, and these were found to be 10% per hour and 5 hours respectively. A similar analysis of the (^{32}P) labeled species could not be made since greater than 50% of the (^{32}P) label existed in peak I as early as T_1 , and peak I contained other non-nucleic acid materials labeled with (^{32}P).

MAK column analyses of the sporulation RNAs show that the (^{32}P) RNA species do chromatograph in the 16S-23S region of the column and may be composed of ribosomal RNA species. In addition, vegetative RNA is turning over so that 50% of the pre-existing srRNA was replaced by newly synthesized species made during sporulation. This new synthesis maintained the bulk RNA at the plateau levels observed in sporulating systems (see Figures 3 and 5). These results suggest that srRNA synthesized during sporulation replaces the vegetative species.

NUCLEIC ACID HYBRIDIZATION AND COMPETITION ANALYSES

The formation of a DNA/RNA hybrid is a very sensitive method used to detect similarities and differences between two sources of nucleic acids. The conditions under which the experimenter examines hybrid formation greatly influences the results obtained. Only 0.4% of the total B. subtilis genome codes for ribosomal RNA sequences (Margulies et al., 1970a). The differential stabilities of m RNA and srRNA, and the higher frequency of rRNA cistrons in the B. subtilis chromosome, i.e. 6-10 copies of each 16S and 23S gene (Smith et al., 1968), can account for the relative abundance of the stable RNA species, e.g. greater than 95% of the RNA isolated from a cell (Midgley, 1969). Since such disparate proportions exist between the quantity of rRNA present in the cell and the small number of rRNA cistrons in the genome, a large excess of DNA must be used to hybridize all the available ribosomal RNA. The use of single stranded DNA in the hybridization reaction removes the possibility of competition between the RNA and the homologous DNA strand. It has been shown previously by Oishi (1969) and Margulies et al. (1970a) that 16S, 23S and all soluble RNA, i.e. 4S + 5S, are transcribed exclusively from the pyrimidine rich H-DNA strand (Rudner et al., 1972) of B. subtilis. Therefore to accurately titrate all of the srRNA sequences in a RNA population, a great excess of H-DNA must be used. The choice of the experimental procedures employed in these analyses and the sources of nucleic acids will

be discussed here.

Strand Separation of DNA

Denatured B. subtilis DNA chromatographed on a MAK column as described in the Materials and Methods, was separated into two complementary strand populations designated L and H, by use of the intermittent gradient technique devised by Rudner et al. (1968b; see Materials and Methods). Figure 17 presents a typical MAK elution profile of denatured Bacillus subtilis DNA. Each single stranded DNA preparation was pooled individually. The pooled L or H DNA populations from numerous MAK columns of the same DNA preparation served as the preparations of DNA in the hybridizations reactions described. This pooling procedure was employed in order to minimize the variability in the quantity of hybrid formed when different DNA strand pools were used (Margulies et al., 1970a). The DNA from each bacterial strain used in these studies, was isolated, strand separated and used to hybridize the homologous cellular RNA species synthesized by that same bacterial strain (see Figure 17).

Large quantities of single stranded DNA were required in hybridization experiments employing conditions of DNA excess, i.e. DNA/RNA of 75:1 to 200:1. Care was taken to pool the individual DNA strand populations without cross contamination of the complementary DNA strand which would compete effectively with the RNA for similar homologous sites (Margulies et al., 1970a).

Chromatography of Stable RNA

Stable RNA from Bacillus subtilis A26u⁻ cells at different stages of growth and sporulation was prepared using rifampicin (30 ug/ml and 100 ug/ml respectively) or Actinomycin D (10 ug/ml for 10 minutes) and isolated. Ribosomal RNA (rRNA) was prepared from purified 30S and 50S vegetative ribosomal subunits isolated from strain A26u⁻ after incubation with rifampicin (30 ug/ml for 20 minutes). The chromatographic elution profiles of ribosomal RNA and stable RNA (srRNA) from a MAK column are shown in Figures 18 and 19 respectively. The profile of rRNA exhibited the following properties: (1) lack of low molecular weight material eluting before and with the salt gradient, i.e. peak 1, and (2) the large quantity of 4S transfer RNA present in the srRNA profile (compare Figures 15, 18 and 19). The unlabeled rRNA and srRNA preparations were used as competitors with the radioactive vegetative or sporulation RNAs. When these unlabeled stable preparations, i.e. rRNA and srRNA, were used in competition with the labeled RNA, the quantities of tRNA, rRNA and mRNA in the radioactive RNAs could be determined. Table 5 presents the results of an experiment designed to determine the most suitable DNA/RNA ratio that would quantitatively bind the Actinomycin D-chased srRNA present in a reaction. A DNA/RNA ratio of 200:1 resulted in hybridization of greater than 99% of the input srRNA. In addition, greater than 98% of the srRNA bound at all DNA/RNA ratios was competed with unlabeled vegetative or sporulation competitor srRNA. It is evident from these results that none of the stable RNA species bound to the L-DNA strand and that which bound to the H-DNA strand was coded for by only a small portion

of the bacterial genome, (see Table 5). Therefore, an accurate determination of the relative proportions of rRNA in a mixed population of RNA could only be established under saturating conditions. At any point below this saturation level, all of the mRNA species was bound but only a portion of the more frequent srRNA species was hybridized.

A DNA saturation experiment was performed for each radioactive RNA preparation to determine the quantity of DNA which must be used for saturation. Typical results of such experiments are presented in Figures 20 and 21, for the RNAs extracted from sporulating and asporogenous cells respectively. *B. subtilis* A26u⁻ cells were pulse-labeled for 3 or 8 minutes during vegetative growth in Spizizen minimal salts medium for in SM medium, and their respective RNAs extracted and hybridized with increasing H and L DNA inputs (see Figure 20 A and B). Similar experiments were performed with the same cells doubly labeled with (³H)Uridine for four generations during vegetative growth and (³²P)phosphate during sporulation, for 30 minutes in SM-R media. (see Figures 20 C & D and 21). The results presented in Figures 20 and 21 demonstrate the following: (1) a large excess of pooled DNA, e.g. DNA/RNA of 100 to 200:1 was needed to maximally bind the input radioactive RNA, (2) greater than 85% of the pulse-labeled RNA (Figure 20 A & B) and all of the stable RNA (the (³H)label in Figures 20 C & D, and 21) hybridized exclusively with the H-DNA strand, and (3) 2 to 8% of the hybridizable RNA hybridized with the L-DNA strand pools. These quantitative differences between the H and L DNA strands, with respect to

hybridization, were observed throughout these studies.

Quantitative differences refer to the relative abundance of L or H specific transcripts in an RNA population, which due to their frequency, e. g. rRNA, required a greater quantity of homologous DNA for maximum hybridization. Qualitative differences refer to the different gene transcripts, irrespective of their relative frequency. Qualitative differences, which will be discussed later, were assayed under conditions of RNA excess and approaching saturating levels of RNA, if experimentally possible.

The greater abundance of RNA transcripts hybridizing to the H-DNA strand, i.e. tRNA and rRNA (and mRNA?) was also evident when radioactively labeled RNA was hybridized to individually pooled regions of B. subtilis DNA as it eluted from a MAK column. RNA was extracted from cells of strain A26u⁻ labeled for three minutes with (³H)Uridine in SM-R media during vegetative growth (SM) or stage T₂ of sporulation (R). Figure 22 presents the quantities of RNAs which hybridized to the DNA under conditions of excess DNA, i.e. DNA/RNA of 75:1. It was previously shown (Rudner et al., 1968b; Margulies et al., 1970 a,b; Galloway, 1975) that genetic markers, and vegetative, ribosomal and messenger RNAs of Bacillus subtilis are preferentially fractionated in the late eluting region of the H-DNA strand, as it eluted from a MAK column. This skewed characteristic of the H-DNA strand was also evident in the quantitative hybridization of vegetative and sporulation transcripts (see Figure 22). This MAK fractionation of L and H DNA demonstrates

that the greater majority of mRNA and srRNA species synthesized during growth and sporulation are transcribed from the genes eluting in the H DNA region of the MAK profile. The exaggerated hybridization of the vegetative RNA was probably due to the two-fold greater abundance of rRNA transcripts compared with sporulation T_2 RNA, which will be demonstrated later. Ribosomal RNA was shown to hybridize more efficiently with the late eluting H-DNA region (Margulies et al., 1970a) and may influence the observed hybridization profile. The quantitative hybridization of messenger RNA species with denatured DNA eluted from the MAK column presented in Figure 22 was examined by correcting for quantity of ribosomal RNA present in each labeled RNA preparation by hybridization-competition analyses (see legend to Table 6). These results as presented in Table 6, demonstrate the following: (1) the late eluting region of H strand DNA (a) contains the same vegetative cistrons found in earlier eluting DNA regions (compare the amounts of input vegetative RNA hybridized with the DNA pools I, J and K), and (b) lacks approximately 20% of the T_2 sporulation genes which elute in earlier fractions of the MAK profile, (2) larger quantities of mRNA are transcribed from the L DNA strand at T_2 of sporulation than during vegetative growth, and (3) a high proportion of T_2 sporulation mRNA transcripts hybridize to the early eluting region of H strand DNA. This data suggests that the B. subtilis genes transcribed at different stages of development are fractionated in separate regions of the DNA MAK elution profile. Much less RNA was

observed to hybridize with the L-DNA strand, e.g. 2% to 8% a characteristic accentuated by the DNA/RNA hybridization ratio chosen in these studies, i.e. high DNA/RNA ratios. At high RNA/DNA input ratios (see Figure 25) which will be discussed later, the difference in hybridization between the two strands was not as large as presented here in Figures 20 and 21. There was a consistent and repeatable increase in the quantitative hybridization of mRNA to the L-DNA strand when comparing sporulation and vegetative transcripts (see Figure 22). These results were observed throughout these studies and will be discussed later.

Hybridization Properties of Sporulation RNA

The RNA isolated from sporulating cells appears to be different in its hybridization efficiency compared to the RNA isolated from vegetative cells. Virtually all, i.e. 98%, of the vegetative RNA species hybridized to the DNA whereas the quantity of hybridizable sporulation RNA was considerably reduced under the same saturation conditions (see Figures 20, 21 & 22). This reduced hybridizability of the sporulation transcripts appeared to be a specific characteristic of these stationary phase transcripts since vegetative srRNA isolated from cells doubly labeled did not show this decrease (see Figures 20 and 21). Pero et. al., (1975) have reported a lower efficiency, e.g. 53 to 62% of the input RNA, of hybrid formation for their sporulation transcripts assayed under conditions of DNA excess.

The decrease in quantitative hybridization in stationary phase

cells, of which strain OA5NA is an extreme example (see Figure 21) could have been the result of the following: (1) secondary structure in the stationary phase RNA, which would result in the selfannealing of that RNA and therefore prevent its hybridization to the homologous region on the DNA, or (2) selection of the L and H DNA strand pools used in the hybridization assays which excluded some of the sporulation or stationary phase genes.

The first possibility was eliminated when a mock hybridization reaction, in the absence of DNA, was performed. The quantity of RNase-resistant RNA formed under these mock conditions never exceeded 3% of the total input RNA. In addition, the quantity of RNase-resistant RNA present at stage T_2 of sporulation was the same as that found during vegetative growth (results not shown). The second possibility can be examined in view of the results presented in Figure 22. The pooling of the individual DNA strands, e.g. in Figure 22: L pool, tubes 50-60; H pool, tubes 69-76, did not alter the results, namely the sporulation RNA still hybridized less efficiently than the vegetative RNA.

The decreased hybridization efficiency of sporulation RNA preparations reported here and by Pero et al. (1975) remains to be resolved. These experiments were performed to identify whether this observed behavior was due to some artificial bias inadvertently imposed during the analyses of hybrid formation. The hybridization efficiencies of the different radioactive vegetative RNA preparations are very good when compared to the published data, e.g. 32 to 74%

(Segall et al., 1974; Pero et al., 1975). The results presented here suggest that the reduced ability of hybrid formation found in the sporulation transcripts was not due to the assay conditions employed to measure the hybrid formation. Bonamy et al. (1976) have also recently reported that sporulation transcripts have peculiar hybridization characteristics. In their hands, they have found that 100% of the B. subtilis genome can hybridize to the labeled sporulation RNA. This implies that the entire genome in this bacterium is transcribed during sporulation, a characteristic not observed in other laboratories (Dicioccio & Strauss, 1973; Sumida-Yasumoto and Doi, 1974; Testa, Ph.D. Thesis). Bonamy et al. (1976) have been unable to identify these properties.

Hybridization-Competition Analyses of Double Labeled Sporulation Transcripts

Hussey et al. (1971) have proposed that ribosomal RNA synthesis is "turned-off" by T_1 of sporulation, a conclusion drawn from the decreasing amounts of radioactivity found in the 16 and 23S RNAs, and 30 and 50S ribosomal subunits. To accurately measure the quantity of stable RNA synthesized during sporulation, the technique of DNA-RNA hybridization and competition were used in the studies presented here. In double-labeled hybridization experiments, an internal ^3H labeled vegetative stable RNA was used as a control for the assays of DNA-RNA hybridization and competition. The experiments were designed to label the stable RNA of vegetative cells with (^3H) Uridine, induce the cells to sporulate in R medium, and then add to these (^3H) -labeled cells (^{32}P) phosphate at the different stages of sporulation. The RNA extracted from these cells would then contain (^3H) -labeled vegetative stable RNA and (^{32}P) -labeled sporulation RNAs. The hybridization efficiency of each type of RNA could then be evaluated.

The content of stable RNA in a labeled RNA preparation was accurately determined by hybridization-competition experiments using unlabeled srRNA preparations. The stable RNA preparations used as competitor RNA in these studies were prepared from three different stages of development: vegetative growth (V) in a minimal salts medium (Spizizen, 1958) supplemented with 0.02% casein hydrolysate, and two stages of sporulation in R medium which lacks amino acids, $T_{2.5}$ and T_6 (S_I and S_{II} respectively).

Bacillus subtilis strains A26u⁻ (sp⁺), OA5NA (sp⁻) and SP-8-8 (sp⁻) were grown in SM medium in the presence of (³H)Uridine followed by the addition of unlabeled uridine to dilute the labeled isotope. The (³H)labeled cells were resuspended into R medium and labeled with (³²P)-phosphate for 30 minutes. The extracted RNA was purified on a MAK column prior to use in hybridization experiments to remove the material which elutes in peak I (see Figure 15). Each doubly labeled RNA preparation was then mixed with increasing quantities of DNA H strands to determine the level at which maximum hybridization was achieved (see Figures 20 and 21). At this saturation level, the 50 or 100 fold excess unlabeled srRNA added effectively competed all labeled srRNA which could have formed hybrids with the H DNA strand. The results of these experiments are presented in Table 7. Two individually labeled vegetative RNA preparations, isolated from strain A26u⁻, are included in this study as a comparison of the quantities of srRNA present in vegetative cells. Vegetative cells growing in a minimal salts medium (Spizizen, 1958) lacking amino-acids, and in SM medium containing 0.8% casein hydrolysate, were labeled with ³H-Uridine for three and eight minutes respectively. The RNAs isolated from vegetative A26u⁻ cells were quantitatively hybridized to DNA H strands under conditions of DNA excess, i.e. DNA/RNA of 200:1. These radioactive vegetative RNAs, when competed with vegetative (V) or sporulation (S_I) srRNA were found to contain 43 and 65% srRNA respectively (see Table 7, experiments 1 & 2). The remainder of the RNA not competed from forming a DNA-RNA hybrid, was the mRNA fraction specific to H strand DNA.

The double-labeled sporulation RNAs used in these hybridization studies were devoid of the peak I material and therefore represented a selected population of RNAs (see figure 15). The conditions used in these studies, demonstrated that greater than 97% of all the (^3H) label of the sporulating cells, and greater than 98% of this label was found hybridized to the DNA H strand. The (^{32}P)labeled sporulation RNA on the other hand, did not reside solely in the stable RNA fraction, which comprised 28-78% of the label, depending upon the stage of sporulation (see Table 7, experiments 3 & 4). In addition, there was essentially no difference in the quantities of radioactive RNA competed from hybrid formation at each sporulation stage when different sources of unlabeled competitor srRNA were used, i.e. V, S_I , or S_{II} . From these results, it was clear that ribosomal RNA continued to be synthesized during stationary phase in sporulating cells and that this srRNA was not detectably different from that made during vegetative growth. The content of srRNA in (^{32}P) labeled RNAs of A_{260} appeared to be characteristic of the stage of sporulation and related to the overall rates of RNA synthesis demonstrated earlier (see Figure 10). One hour after the cells were resuspended in R medium, only 28% of the newly synthesized RNA was srRNA, analogous to the behavior observed during the glucose shift-down experiments discussed earlier. Once the cells had adjusted to the shift-down conditions by T_2 , an increase in the rates of RNA synthesis and the accumulation of srRNA became apparent. Stages $T_{2-2.5}$ and T_{4-5} were shown to contain 66 and 78% srRNA

which was newly synthesized during sporulation.

If ribosomal RNA synthesis continued during development, did it also continue in cells unable to sporulate, namely in, asporogenous mutant cells and in the phenocopies? Two different asporogenous strains of B. subtilis, OA5NA and SP98-8, were also studied by the double label hybridization-competition technique. Only one stage in stationary phase was examined, $T_{2.5}$. At this point in stationary phase of these spo0 mutant cells, competition experiments demonstrate that 72% of the hybridizable RNA synthesized contained srRNA sequences (see Table 7, experiments 5 & 6). The lower hybridization efficiency of stationary phase (SP98-8 and OA5NA) or sporulation (A26u⁻) RNAs is also shown in Table 7. The decreased hybridization efficiency of the sp⁻ strains (see Table 7, experiments 5 & 6) suggested that this characteristic, as discussed earlier was a result of the stationary phase and not just specific to sporulation.

These results also suggested that the synthesis of stable RNA was not regulated by the events governing sporulation-specific events, but rather was controlled by the factors regulating rRNA synthesis during slowed growth. In conclusion, these results demonstrate that stable RNA and therefore ribosomal RNA, syntheses continues during (1) sporulation of Bacillus subtilis strain A26u⁻, and (2) stationary phase of asporogenous mutant cells. The quantity of stable RNA synthesized is determined by the stage in stationary phase and not the spore genotype of the cells used.

Hybridization-Competition of Pulse-Labeled Transcripts

The duration of exposure of the cells to the (^{32}P) phosphate radioisotope, as discussed above, was considerable and was used to detect the presence or absence of srRNA synthesis during sporulation. The thirty minute labeling probably influenced the quantity of srRNA present in the total RNA synthesized at that time because of the faster turn-over rate of the mRNA sequences (Leighton and Doi, 1971). To determine the quantities of the srRNA synthesized relative to messenger RNA during growth and sporulation, shorter periods of exposure to the radioisotope had to be used and the total population of RNA synthesized, were used for the hybridization and competition analyses.

B. subtilis strain A26u⁻ was grown in the three most commonly used sporulation media, e.g. 2xSG, 121B and SM-R, and one ml of cell culture during different stages of growth and sporulation was mixed with (^3H)Uridine (200-400 uc/ml) for three minutes. The RNA was extracted by the Summers' procedure (Summers, 1969) and hybridized to the fractionated DNA strands. Cold competitor srRNA and rRNA was used to compete with the binding of stable radioactive RNA components in the pulse-labeled preparation. The results of these competitions are presented in Table 8. As shown in this table a large quantity of the stable RNA was synthesized by vegetative cells, i.e. 76 to 89% of the hybridizable RNA. The data presented in Table 8 demonstrate that the three nutrient rich media used, SM, 121B and 2x SG, supported rapid cell growth and high cellular contents of ribosomal RNA. The same cells, when grown

in a nutrient poor medium, grow at a much slower rate and synthesize about half the quantity of rRNA found synthesized in the cells grown in the nutrient rich media (only 43% compared with 76-89%; compare Table 7, expt. 1 with Table 8). On the other hand less srRNA was synthesized during stationary phase in each of the media shown. A gradual decrease in the quantities of srRNA synthesized could be detected during the first two hours in sporulating (sp^+) and non-sporulating ($SpoM_c$) cells. This decrease in the amount of srRNA made during sporulating however, was not as dramatic as the depressed levels reported originally by Hussey et al. (1971), e.g. 90% decrease in rRNA synthesis by T_1 , or in a later report from the same laboratory, e.g. 75% decrease in rRNA synthesis by T_2 (Pero et al., 1975). In addition, A26u⁻ cells, whether grown in a medium which supported sporulation or not, showed only a 40 to 50% decrease in the quantity of srRNA synthesized at T_2 as compared with the amount made during vegetative growth (see Table 8). These results demonstrated that synthesis of stable RNA components was probably not directly involved in the events leading to sporulation, since similar patterns of srRNA synthesis were observed in sporulating (sp^+) and non-sporulating ($SpoM_c$) cells of A26u⁻.

The "turn-off" of rRNA synthesis reported by Hussey et al., (1971) and Pero et al., (1975) was observed in a different strain of Bacillus subtilis, namely strain 3610. It was therefore of interest to determine whether the relatively smaller decrease in the

synthesis of rRNA during stationary phase and sporulation of strain A26u⁻ was a strain-specific behavior. The quantity of rRNA synthesized in strains 3610, W23 and BD29 during growth and stationary phase in 121B medium is presented as part of Table 9. Strain 3610 showed a similar 40 to 50% reduction in the synthesis of rRNA during sporulation, a pattern identical to that observed with A26u⁻. Strains W23 and BD29 both SpoM_C phenocopies in 121B medium, demonstrated very different behaviors with respect to the amount of rRNA synthesized during stationary phase (see Table 9). The behavior of each strain, i.e. W23 and BD29, may be reflective of the overall rates of RNA synthesis observed in this same medium (see Figure 13, panel B & C). Strain BD29, which demonstrated the largest relative decrease in rRNA synthesis at T₂ in 121B medium, i.e. a 72% decrease, also showed corresponding low rates of RNA synthesis during that time (see Figure 13B). Strain W23 however, increased its overall rate of RNA synthesis after an initial rate transition in 121B medium, which correlated with the smaller decrease in rRNA synthesis during that same period. (Compare Table 9, experiments 2 & 3 with Figure 13, panels B & C).

In addition to the sp⁺ and SpoM_C strains studied, the proportion of rRNA synthesis during a three minute pulse label during stationary phase of sp⁻ mutants were also examined. Two asporogenous strains of B. subtilis, a derivative of 168, i.e. SP98-8, and a rifampicin-resistant derivative of strain 3610, i.e. rfm3Y were pulse-labeled with (³H)Uridine in an identical manner as stated above, and the quantity of rRNA in the radioactive RNA determined. The results of these experiments are presented as part of Table 10. Again, a

50% decrease in the quantity of rRNA synthesized at T_2 in 121B medium, relative to the vegetative levels, was observed. Two of these strains, SP98-8 and rfm3Y also showed rate transitions and low rates of RNA synthesis during stationary phase in this same medium. The similarity between the three spore phenotypes studied, sp^+ , $SpoM_c$ and sp^- , with respect to their rates of ribosomal RNA synthesis during stationary phase, demonstrated that this behavior was characteristic of stationary phase cells and not specific to sporulation.

It therefore appeared that ribosomal RNA synthesis during stationary phase was regulated by the decreased growth rate of the cell population. This decreased growth rate could have occurred by the natural depletion (e.g. 2xSG and 121B media) or artificially-induced depletion, (e.g. SM-R media) of some essential nutrients in the medium required for growth. If this was the case, then removal of certain compounds from the growth medium, like magnesium, glucose, amino acids, should reduce the growth rate of the cells with a concomitant reduction in the quantity of ribosomal RNA synthesized. The latter was tested as described below.

1. Reduction of Magnesium Concentration in the Medium

The effect of reducing the magnesium ion concentration and the coordinate decrease in the synthesis of stable RNA has been well documented (Suzuki and Hayashi, 1964; Sykes and Tempest, 1965). When the magnesium concentration in a growing cell culture becomes limiting, stable RNA synthesis will be depressed. In the media used in these studies (see Appendix A) the concentration of this divalent cation ranged from 0.8 to 40 mM in all but one medium, that is 121B.

In that medium, as modified by Hussey et al. (1971), the original concentration of 1.0 mM Mg⁺⁺ ions was replaced by a ten-fold lower concentration, that is 0.1 mM. This decrease in the magnesium ion concentration is sufficient to cause an additional 15% decrease in the quantity of rRNA synthesized during stationary phase of sp+ and SpoM_c cells (see Table 11). The larger decrease in the quantity of rRNA synthesized in medium 121B containing 0.1 mM magnesium, was most evident at T₂ of stationary phase. The lower level of rRNA synthesized by strain 3610 at T₂ in 121B medium containing 0.1 mM magnesium (37%) is still higher than that reported by Pero et al. (1975; 10%) which may be due to the shorter pulse period used in their study.

2. Glucose or Amino Acid Shift-Down

Cells which enter stationary phase have reduced growth rates. Since the reduction of stable RNA synthesis occurred during the transition from growth to stationary phase, it was of interest to study the effects of nutrient deprivation on growing cells. Bacillus subtilis strain A26u⁻ was grown in a minimal salts medium (Spizizen, 1959) supplemented with 1.0% glutamate, 0.5% glucose and 0.5% casein hydrolysate (Medium I). At logarithmic growth an aliquot of cell was centrifuged, washed and resuspended into the original medium lacking glucose (Medium II). The growth of these cells in Media I and II have already been described (see Figure 8, panels A and B). The deprivation of glucose from the growing cells appeared to have a strong effect on the growth rate (μ = generation time) of the cell culture (see Table 11). One milliliter samples of cell cultures in the

different media were mixed with (^3H)Uridine, for three minutes, the RNA extracted (Summers, 1969) and the quantity of rRNA determined. The results are presented in Table 12. Forty-seven percent of the RNA synthesized in the cells growing in the complete medium (Medium I) contained ribosomal RNA transcripts. The small quantity of rRNA, 47%, synthesized in this medium supplemented with glucose and amino acids, was surprisingly low, since the same cells grown in the absence of amino acids contained 43% rRNA (see Table 7, expt. 1). This suggests that these cells were not utilizing the available amino acids provided in the medium. However, a carbon source shift-down as observed in the glucose deprived cultures (Medium II), drastically altered the rate of RNA synthesis (see Figure 8) and the corresponding quantities of ribosomal RNA synthesized, 9% until the cell culture adjusted to glutamate and amino acids as the carbon sources, and growth was resumed.

The content of ribosomal RNA as a function of the growth rates of vegetative cells in different media is presented in Figure 23. The content of rRNA in a three minute pulse-labeled RNA preparation was determined by hybridization-competition experiments as described above. It was calculated from these measurements that there was a strong positive correlation (see Appendix B), i.e. the correlation coefficient, r equals +0.89, between the vegetative growth rate of a cell and the cellular content of ribosomal RNA. The quantity of ribosomes and tRNA molecules have also been shown to be directly proportional to the cellular growth rates by Kjeldgaard and Gausing (1975).

The artificial shift-down experiment presented, was analogous to the events occurring in the transfer of cells from SM growth medium to the sporulation resuspension medium (R). In both cultures, the glucose shift-down and the SM-R transfer, there was a rapid decrease in the rate of RNA synthesis (see Figures 8 & 10) and the quantities of ribosomal RNA synthesized (see Tables 6 and 11), which were seen to increase as the cells adapted to the new culture media.

The sporulation media used in these studies, 121B, 2xSG and SM-R, are rich in the nutrients which supported active cell growth. Vegetative cells pulse-labeled during growth in these media were therefore found to contain very high quantities of rRNA, i.e. 70 to 90% (see tables 7, 9, & 10), when compared with cells grown in less enriched media, i.e. 43 to 47% (see Table 7, expt. 1 and Table 12 expt. 1). The relative quantities of ribosomal RNA made during different sporulation stages was normalized to the quantity of rRNA synthesized in the vegetative cells of the same medium, e.g. 121B and 2xSG, or the previously used growth medium, e.g. SM. Therefore the quantity of rRNA synthesized during stationary phase in 121B and 2xSG media was regulated by a naturally occurring nutrient depletion when compared to the SM-R transfer which induced an artificial nutrient depletion. In conclusion, the relative decrease in the amount of ribosomal RNA synthesized in all three sporulation media at T_2 relative to the vegetative quantity of rRNA, is essentially identical and unrelated to the sporulation capacity

of the strains used in these studies.

THE EFFECTS OF CHLORAMPHENICOL ON RIBOSOMAL RNA SYNTHESIS

As discussed in the Introduction, the control of rRNA synthesis during growth rate transitions appears to be regulated by the levels of the two magic spot nucleotides (MSI and MSII; Cashel and Gallent, 1975) although this has recently been contradicted (Khan and Yamazaki, 1974; Hansen et. al., 1975). The synthesis of rRNA has been shown to be dependent upon protein synthesis and can be stimulated in slow growing cells treated with chloramphenicol (CM) (Kurland and Maaloe, 1962). Segall et al., (1974) have demonstrated the chloramphenicol-sensitivity of an unstable "sigma factor inhibitor" which is present in sporulating cells by T_2 . A brief treatment of stationary phase or sporulating cells with chloramphenicol was reported to (1) restore vegetative sigma factor activity which was lost during sporulation (Segall et al., 1974), and (2) increase the quantity of ribosomal RNA synthesized in sp^+ and sp^- cells of Bacillus subtilis (Pero et al., 1975). The quantity of rRNA synthesized in B. subtilis, irrespective of the stage of growth or development, appears to be governed by the same controls affecting rRNA transcription during shift-down transfer conditions (see Tables 11 & 12). The "sigma factor inhibitor" identified by Segall et al., (1974) may be involved in the regulation of ribosomal RNA synthesis during all stages of growth and development (Pero et al, 1975). Therefore the effects of chloramphenicol on messenger, transfer and ribosomal RNA

synthesis in three cell types, sp^+ , sp^- , and $SpOM_c$, grown in the different sporulation media was studied here.

1. Glucose Shift-Down

Cells of Bacillus subtilis strain A26u⁻ grown in a glucose containing medium (Medium I) were shifted to an identical medium in the presence or absence of chloramphenicol (CM; 200 μ g/ml) and lacking glucose (Medium II) as described previously (see Figure 8 and Table 12). The rates of RNA synthesis in the presence and absence of CM for these glucose deprived cells, were determined. As demonstrated by Figure 8B, the presence of the drug during the entire 45-55 minute lag phase did not increase the rate of RNA synthesis relative to the untreated cells. The addition of CM from the time of resuspension (R_0) throughout the experiment, also prevented the adjustment of these glucose deprived cells to the new growth medium as compared to the untreated cells. This inability to adjust to the medium without glucose was evident from the decrease in cell mass and the decreased rates of RNA synthesis during the new log phase. In light of these results, the pulse-labeled RNA to be used in the hybridization assays was extracted from cells that were briefly pretreated with CM (200 μ g/ml) for only twenty minutes during the adjustment (lag; from time zero to 20 minutes) or growth (log) phases, before the addition of the (^3H)Uridine. The pulse-labeled RNAs were extracted from the cells as described previously and the quantities of rRNA present in the CM-treated cultures was determined. The results of these experiments are presented in Table 12, experiments 2A and 2B.

During the adjustment period (lag) when the cellular growth rate was almost zero, the pretreatment of the cell culture with CM prevented the depression of ribosomal RNA synthesis, i.e. -CM = 9%; +CM = 62.5%. The large quantity of rRNA made in the CM-treated cells during the lag phase (see Table 12, expt. 2A), exceeded the levels of rRNA synthesized in the original culture containing glucose (see Table 12, expt. 1). This result suggests that cellular synthesis of ribosomal RNA in Medium I (see Table 12, expt. 1) was maintained below a potentially high level by a chloramphenicol-sensitive event. A similar response with respect to rRNA synthesis in the presence of CM was also observed in the same cells during the growth phase (see Table 12, expt. 2B). In both experiments the quantities of rRNA synthesized after the cells were pretreated with CM, were virtually identical, i.e. 62.5% and 64%, even though the respective untreated cultures differed greatly in the quantities of rRNA synthesized, i.e. 9% and 40.6%. The quantitative hybridization of RNA to the L DNA strand which is also presented in Table 12, will be discussed in a separate section.

In conclusion, these experiments have demonstrated that ribosomal RNA synthesis in vegetative cells is regulated by a chloramphenicol-sensitive process(es) which limits the quantity of rRNA transcribed in Bacillus subtilis cells during growth and adjustment to a new growth rate. The involvement of the phosphorylated nucleotides (MS) will be discussed later.

2. Stationary Phase and Sporulation

Rhaese and his coworkers (1974, 1975, 1976) have reported that prior to stationary phase in sporulating cells, there is an accumulation of MS and HPN nucleotides. The increased quantity of these phosphorylated nucleotides at T_0 and beyond coincides with the decreased rates of RNA synthesis in sporulating and non-sporulating cells (see Figures 10, 11, 12, & 13). MS nucleotides are implicated in the regulation of rRNA synthesis in vegetative cells, and since they appear between T_0 and $T_{1/2}$ of the transition period (Rhaese et al., 1976), they may initially function to depress rRNA synthesis during that period. The effect of Chloramphenicol in growing and non-growing cells was studied.

The effect of CM on the uptake of (^3H)Uridine in vegetative and sporulating cells of Bacillus subtilis is presented in Figure 24. As shown in this figure, the rate of RNA synthesis during vegetative growth in 121B medium in the presence or absence of the drug is identical for the first 20 minutes, after which the CM-treated cells show decreased rates of RNA synthesis (Figure 24A). These results are very similar to the drug-induced effects observed in cells growing in a medium lacking glucose (see Figure 8B). The block of protein synthesis with the addition of the drug (CM) appeared to be more inhibitory to actively growing cells than it was to non-growing or slower growing cells. There was however, a strong stimulatory effect of CM on sporulating cells at stage T_2 or sporulation (see Figure 24B) which was in contrast to the changes induced by this drug in growing cells. In addition, it will be demonstrated that this drug pre-treatment increases the quantity of rRNA synthesized at T_2 . B.

subtilis strains 3610 in 121B medium (Figure 24) and A26u⁻ in R sporulation medium (not presented) exhibited an 8-10 fold increase in the quantities of (³H)Uridine taken up at T₂ by cells pretreated with chloramphenicol at that stage of sporulation. This increase in (³H)-TCA precipitable counts in RNA may also represent the facilitation of uridine into UTP demonstrated by Segall et al. (1974).

The CM drug-induced changes in the specific syntheses of stable RNA transcripts during vegetative growth and development (sporulation) were studied in the three cell phenotypes presented in this study, and the results are presented in Tables 13 and 14. Chloramphenicol added to vegetative cells growing in SM and 121B media did not increase rRNA synthesis relative to the untreated cell cultures as it did in the slower growing cells (see Table 12, expts. 2A and 2B). This result is in contrast to the results obtained when cells are grown in a minimal salts medium (Medium I; Spizizen, 1959) supplemented with glutamate and amino acids (see Table 12). The very high quantity of ribosomal RNA present in cells grown in the enriched media (121B and SM) was taken to represent the upper limits of rRNA synthesis within the cell during a three minute pulse-label because of the (1) inability of CM to increase the absolute quantity of the rRNA beyond the levels observed in untreated cells, and (2) relatively similar quantities of rRNA present in the slower growing drug-treated cells (see Table 12 expt. 2) and those cells grown in the more enriched media (see Table 13).

The depressed levels of ribosomal RNA synthesis evident in cells of A26u⁻ and 3610 sporulating in the SM-R media (see Table 13, expts. 1 & 3) were not completely relieved by the addition of CM.

Instead of the observed 50% reduction in rRNA synthesis at T_2 , there was only a 35% reduction when the same cells were pretreated with CM. These results indicated that some other mechanism must also regulate the levels of rRNA synthesis during sporulation other than the reversible-chloramphenicol stop observed in vegetative cells (see Table 12). The same two strains of B. subtilis mentioned above were also grown in the Sonenshein and Roscoe medium (121B). In this medium, strain 3610 sporulated and A26u⁻ behaved as an asporogenous phenocopy (SpoM_C). Again, the rates of rRNA synthesis during T_2 of stationary phase in 121B medium was seen to be only 50% of the vegetative value, and CM only partially restored this level to 65% of the vegetative level (see Table 12, expts. 2 & 4). It was of interest to examine whether this partial restoration of rRNA synthesis was also observed in two genetically asporogenous (sp⁻) cells of Bacillus subtilis, SP98-8 and OB3Y, in the same two sporulation media discussed above.

The quantity of rRNA synthesized during vegetative growth and stationary phase of two sp⁻ cell lines in the SM-R and 121B media, are presented as part of Table 14. The 50% decrease in rRNA synthesized in stationary phase and sporulating cells at T_2 was also apparent in both sp⁻ strains used and in both media. The decrease in rRNA synthesis observed at T_2 in stationary phase cells was not specific to the spore genotype or phenotype of the strains used in these studies. However, a complete restoration of the vegetative quantities of rRNA present was observed when these cells were pretreated with chloramphenicol. This was the first indication that

some difference between sp⁺ and sp⁻ cells with respect to their regulation of ribosomal RNA synthesis during stationary phase. Apparently the lesion present in the sp⁻ cell genome was sufficient to cause these cells to behave similarly to vegetative cells with respect to the large amounts of rRNA synthesized after chloramphenicol treatment (compare Table 14 with Table 12, expts. 2A & B).

In conclusion, the quantity of ribosomal RNA in stationary phase cell cultures is observed to decrease to 50% of the corresponding vegetative levels, irrespective of the sporulation capacity of the strains used. A discernable difference does exist however, between sp⁻ and sp⁺ cell genotypes, in the extent to which the depressed rates of rRNA synthesis can be reversed. Asporogenous cells (sp⁻) maintain the ability to return to the full vegetative rates of ribosomal RNA synthesis, whereas sp⁺ and SpoM_c cells have modified this potential by stage T₂ of stationary phase.

MESSANGER RNA TRANSCRIPTION

An early report by Yamakawa and Doi (1971) demonstrated that although both L and H DNA strands were transcribed during sporulation, there was a preferential increase in the transcription of messenger RNA from the L strand of Bacillus subtilis DNA at stage T₄ of sporulation. More recent studies by DiCioccio and Strauss (1971; 1973) and Sumida-Yasumoto and Doi (1974) have shown that in addition to new transcription from the L strand, sporulation-specific messenger RNA transcription also occurred from the H strand. The above mentioned studies have analyzed the qualitative differences between the messenger RNA populations of developing cells. The studies presented here were

designed to examine the quantitative changes occurring in the messenger RNA populations which were transcribed from the L strand of B. subtilis DNA at different stages of sporulation.

The quantities of mRNA hybridizing to L DNA under conditions of DNA excess is very much smaller than that hybridizing the H DNA under similar assay conditions, i.e. less than 8% (Margulies et al., 1971b; see Figures 20 and 21). The large quantity of L DNA for hybridization and the small proportion of the input RNA which hybridizes to the L DNA, limited the extent to which the quantitative analyses of L-specific mRNA could be analyzed in these studies. In the results presented here, the saturation of L DNA with RNA occurred at a DNA/RNA ratio of 100:1 (see Figures 20 and 21), and this ratio is the one used unless otherwise stated.

The quantity of L-specific messenger RNA present in a cell appeared to be related to the nutrient enrichment of the culture medium in which the cell was grown. A reexamination of Table 12 shows that as much as 6 to 8% of the total RNA synthesized in a supplemented minimal salts medium was specific to the L DNA strand, whereas only 1 to 3% were L-specific vegetative transcripts in the RNA isolated from cells grown in more enriched media (see Tables 9, 10, 13 & 14). The quantity of mRNA synthesized from the L strand seemed to be regulated by a CM-sensitive mechanism. In all but one experiment, i.e. experiment 2B of Table 12 the pretreatment of a growing cell culture with CM, increased the quantity of L-specific mRNA synthesized during a three minute pulse-labeling (see Tables 12 and 13). Three to five percent of the input pulse-labeled RNA hybridized to the L strand of B. subtilis DNA after treatment of the cells with

CM. This proportion was considerably higher than the values observed in the untreated cell cultures, i.e. 1 to 3%, but not as high as that observed in the supplemented minimal salts medium, i.e. 8% (see Table 12, expt. 2B).

The quantity of L-specific transcripts detectable by DNA-RNA hybridization was also observed to increase as the growing cells slowed their growth rates during sporulation and stationary phase (see Tables 13 and 14). As demonstrated with vegetative cells, the addition of CM to stationary phase cultures of sp^+ cells increased the quantity of L-specific mRNA transcripts present at T_2 . In contrast to the chloramphenicol-induced accumulation of L-specific mRNA transcripts, was the behavior of the asporogenous cells treated at T_2 of stationary phase with CM (see Table 14). In all of the sp^- cells pulse-labeled with (^3H) Uridine after pretreatment with CM, there was a decrease in the quantity of L-specific mRNA hybridizable to the L strand (see Table 14). This apparent decrease in the quantity of L-specific mRNA however, was due to a dilution of these transcripts by the very high levels of rRNA synthesis in these sp^- drug-treated cells. When the quantities of RNA synthesized in the presence and absence of CM are adjusted for their messenger RNA contents, by correction for the rRNA content present, no decrease in L-specific transcripts were observed. The average quantitative increase in the L-specific messenger RNAs, at T_2 in the drug-treated cells, was 4.1% and 0.3% for the sp^+ and sp^- cells respectively. These results demonstrate that the sp^+ cells are stimulated in the presence of CM to increase the quantities of L-mRNA, while the sp^- cells are unaffected in this respect. This

suggests the presence of an inhibitor of RNA synthesis present only in the $\underline{sp}^+$ cells, an idea which will be discussed later. In conclusion, the quantity of L-specific mRNA synthesized was found to be related to the following: (1) the nutrient enrichment of the culture medium and (2) the depletion of nutrients in the medium which occurs during growth. In addition, L-mRNA is stimulated in $\underline{sp}^+$ cells and unaffected in $\underline{sp}^-$ cells by pretreating stationary-phase cultures with CM.

The relative quantities of the different RNAs synthesized during vegetative growth and stationary phase of Bacillus subtilis strain A26u⁻ ($\underline{sp}^+$) is presented in summary form in Table 15. This table demonstrates that (1) although very little transcription occurs from the L DNA strand during vegetative growth in the enriched media, there is a two-fold increase in this relative amount as the cells enter stationary phase, (2) the quantity of rRNA synthesized during stationary phase decreases irrespective of the sporulating state ($\underline{sp}^+$ or $\underline{SpoM}_c$) of the cell culture, and (3) chloramphenicol has a dual effect (a) it partially relieves the depressed levels of rRNA synthesis at T_2 of stationary phase, and (b) it increases the quantity of L strand specific mRNAs detectable by the hybridization assay. It is also interesting to note that the quantity of transfer RNA was also observed to increase as the cells entered stationary phase (Doi, 1965) and that this increase was reversed by pretreating the cells with chloramphenicol at stage T_2 .

RNA SYNTHESIS IN SpoM_C CELLS

The results presented by DiCioccio and Strauss (1971; 1973) and Sumida-Yasumoto and Doi (1974) have demonstrated that differences between the messenger RNA populations of vegetative and sporulating cells can be detected. Bacillus subtilis strain A26u⁻ in 121B medium does not sporulate, e.g. SpoM_C, but does maintain some of the regulation over RNA synthesis that is typical of a sporulating cell, i.e. (1) regulation of rRNA synthesis during stationary phase in the presence and absence of CM, and (2) regulation of L-specific mRNA transcription in the presence and absence of CM. If the decreased levels of rRNA synthesis during stationary phase are regulated by an inhibitor of the RNA polymerase sigma subunit, then the treatment of stationary phase cells with chloramphenicol should prevent the synthesis of the sigma subunit inhibitor (Segall et al., 1974). The restoration of the vegetative sigma subunit activity should then be apparent by the (1) increase in the quantity of rRNA transcription and (2) increase in vegetative messenger RNAs detectable in these chloramphenicol-treated cultures.

In order to study the messenger RNA species in a mixed population of pulse-labeled RNA, DNA-RNA hybridization experiments must be performed under conditions which favor the binding of the many more messenger RNA species, i.e. high RNA/DNA ratios. B. subtilis strain A26u⁻ was grown in medium 121B and labeled during vegetative growth

and T_2 for a three minutes with (^3H)Uridine. The cell culture at a point 20 minutes prior to T_2 , was split in half, and one portion was treated with chloramphenicol (200 $\mu g/ml$) for 20 minutes and then labeled at T_2 in the same manner as the untreated cell culture. The cells were pelleted, the respective RNAs extracted and used in various hybridization and competition experiments at different RNA/DNA ratios.

The pulse-labeled RNA was hybridized to fractionated DNA strands under conditions of RNA excess. The relative hybridization of the L and H DNA strands to increasing inputs of labeled RNA is presented in Figure 25. It is evident from this figure, that even at different hybridization ratios, the H strand of B. subtilis still hybridizes the greater amount of RNA. In this type of experiment, i.e. high RNA/DNA ratios, the quantity of rRNA hybridized is low since the limiting factor in this assay is the DNA. The ratio of the radioactive counts bound to each DNA strand under conditions of RNA excess is referred to as the assymetry ratio (Margulies et al., 1971b), i.e. H/L, and represents the relative quantities of different mRNA transcripts copied from each strand of DNA. The quantity of RNA bound (in μg) to 100 μg of DNA, calculated from the values obtained in hybridization experiments employing conditions of RNA excess, is an estimate of the relative portion of the bacterial genome which was actively involved in transcription during the developmental stage studied. The relative amounts (as percent) of DNA transcribed is presented for sporulating (sp^+) and non-sporulating ($SpoM_c$) cells

in Table 16. The results of similar studies with B. subtilis cells sporulating in 2xSG medium reported by Jeng and Doi (1974) are presented as a comparison. These results demonstrate that a smaller portion of the genome was transcribed during vegetative growth than sporulation or stationary phase, and that the quantity of the genome transcribed during growth varies greatly with the medium used. Also demonstrated was the increase in the portion of the genome transcribed, and hence the number of genes "turned-on" as the cells were shifted from the nutrient rich SM growth medium to the nutrient poor resuspension (R) medium (see Table 16). These results demonstrate an increase in mRNA synthesis and substantiate the results presented in Table 15 for the RNA isolated from the same stages but quantitatively assayed under conditions of DNA excess. In contrast to these results were the findings of B. subtilis cells grown in 121B medium. Although a considerable portion of the genome was transcribed during vegetative growth, 8.92% (7.33 + 1.59%) a significantly smaller portion of the genome was transcribed during stationary phase, 6.31% (5.23% + 1.08%) representing a 30% decrease. Since it was previously demonstrated that these SpoM_c cells are blocked at or before the earliest stage of sporulating (see Figure 7), the typical induction of certain sporulation-specific genes may be suppressed, while the naturally occurring "turn-off" of some vegetative functions begins. These cells were presumably transcribing only those genes necessary for the maintenance of the stationary phase condition since they were unable to grow. The similarities

and differences between populations of RNA transcripts was measured by the assay of DNA-RNA hybridization and competition experiments. Competition experiments performed at high DNA/RNA ratios were used to detect quantitative differences in the mRNA populations. High RNA/DNA ratios were used to detect differences between the messenger RNA populations present at different stages of development or when cells are treated with chloramphenicol.

Competition-Hybridization Experiments

1. Excess DNA

Competition experiments were performed with excess DNA to quantitatively hybridize the maximum amounts of the input RNA. Under these conditions of excess DNA, i.e. D/R of 100 to 150:1, the unlabeled competitor RNA never reduced the binding of the labeled RNA to the levels observed with the same RNA species at the reverse ratios, i.e. high RNA/DNA ratios (see Figures 28 through 31). This inability to compete with all of the labeled RNA was also observed when the unlabeled RNA was used in great excess, e.g. 800x the labeled RNA input. This was presumably due to the (1) RNA species present at different frequencies in a population, (2) great number of DNA sites with which the labeled or unlabeled RNA could bind, and (3) the enormous quantities of unlabeled RNA that would be necessary to effectively compete with all of the labeled RNA. The relative frequency of an RNA species in a total population of RNAs, can be estimated from the quantity of homologous RNA it competes with during a hybridization. The stable RNA species is an example of a very frequent class of RNAs. The

kinetics of competition of (^3H)labeled stable RNA in a mixed population of doubly-labeled (^3H , ^{32}P) RNAs, which have been described previously (see Table 6, expt. 4, and Table 16, SM-R media), is presented in Figure 26, panels C and D. Since the srRNA species are a high frequency transcript population, they saturated their available DNA sites and were therefore competed against more effectively at these DNA/RNA ratios, than the non-saturating less frequent transcripts, e.g. L-specific mRNA in Figure 26. In this figure, the (^3H) label in panels A and B represented a heterogeneous RNA population. The RNA in panel B however, was competed against more effectively than the vegetative RNA presented in panel A, presumably because of the higher proportion of srRNA present in the longer labeled vegetative cell transcripts (see panel B of Figure 26 and Table 6 expts. 1 and 2).

The competition of labeled RNA by its homologous unlabeled RNA appeared more efficient in the H-specific transcripts during vegetative growth and stage T_2 of sporulation. The L-specific mRNA during this same period did not contain very many frequent sporulation RNA species until stage T_4 . At that time, a greater quantity of the hybridizable sporulation mRNA could be competed from hybridizing with the L strand of DNA than at the previous sporulation stage, i.e. T_2 (see Figure 26 panel D) suggesting that an increase in the frequency of a certain class of L-specific mRNA occurred at that time.

The radioactive RNA isolated from SpoM_c cells of strain A26u⁻

in 121B medium was hybridized to excess fractionated DNA i.e. DNA/RNA of 100 to 150:1, and competed with the homologous unlabeled RNA at increasing inputs of unlabeled RNA (see Figure 27). The vegetative RNA, which contained the greatest portion of ribosomal RNA (see Table 15) also demonstrated the more effective H-DNA strand competition with its unlabeled RNA (compare panel A with B & C). The preincubation of cells with CM at T_2 , which has been shown to increase the quantity of rRNA also showed a larger decrease in the quantity of pulse-labeled H-specific RNA remaining hybridized after competition with the homologous unlabeled RNA (compare panels B and C of Figure 27). The effective quantitative increase in L-specific mRNA transcription induced by CM, was also evident from these homologous competitions (Figure 27, panels B and C). In the absence of CM, only 15% of the L mRNA was present as a frequent class of RNA, whereas in the presence of CM 30% of the messenger hybridized to the L strand represented the frequent RNA class. This relative increase in the transcripts induced by chloramphenicol will be shown in the next section to represent only an increase in quantity and not the type of L-mRNAs synthesized.

2. Excess RNA

The relative quantitative changes in the overall L and H specific RNA populations as demonstrated in Figures 26 and 27 was determined under conditions of DNA excess. The accurate determination of qualitative differences between the mRNA species made at various periods of development or under the influence of CM however, required that the competition experiments be performed using conditions of RNA

excess, e.g. RNA/DNA of 10:1 to 100:1. Radioactive RNA was hybridized to the fractionated DNA strands in the presence of increasing amounts of unlabeled homologous (same stage) or heterologous (different stage) RNA as competitor. Figure 28 demonstrates these competition experiments performed with the ^3H -labeled vegetative RNA, i.e. Log- (8), and double labeled (^3H , ^{32}P) and sporulation, i.e. stage T_4 , identified earlier (see Table 7 and Figure 26). In these experiments low RNA/DNA ratios were used (10:1). Panels A and B of Figure 28 show that sporulating cells at stages T_2 and T_4 do not transcribe a considerable portion of the vegetative genome. At stage T_4 (see Figure 28, panels C and D) smaller differences exist.

Similar competition-hybridization experiments were performed RNA isolated from strain A26u⁻ growing in 121B medium. These results are presented in Figures 29, 30 and 31. The labeled transcripts were hybridized to the DNA at a high RNA/DNA ratio of 100:1 and mixed with increasing quantities of homologous or heterologous unlabeled competitor RNA. Figure 29 demonstrates that stationary phase T_2 cells, whether preincubated with CM or not, do not contain all of the H-specific mRNAs (panel A) transcribed during vegetative growth. The differences in the competitions to the L DNA strand (panel B) were not as large and suggested that there was some similarity between these transcript populations. The H-specific mRNA populations synthesized at T_2 however, was virtually identical in the CM-treated and untreated cells. Vegetative cells contain almost all of these stationary phase H-specific mRNA sequences (see Figure 30, panel A). During stationary phase, at T_2 , there were populations of H-specific and L-specific mRNAs synthesized which were not present in vegetative

cells (see Figure 30). These populations of stationary phase specific mRNA transcripts were present in both the drug-treated and untreated cells. It therefore appeared that during stationary phase of strain A26u⁻ in 1218 medium (1) some genes transcribed during vegetative growth were "turned-off", and (2) new genes, not transcribed from the L-DNA strand during growth were "turned-on" at T₂ of stationary phase. The effect of chloramphenicol on the stationary phase transcription as assayed by the technique of DNA-RNA hybridization and competition did not significantly alter the populations of mRNA synthesized at that time (see Figure 31). The transcripts synthesized at T₂ in the presence of CM were virtually identical to those synthesized at T₂ in the untreated cells.

A summary of the competition curves shown in Figures 28 and 29 through 31 is presented in Figure 32. In both sets of competitions, 32A-C and 32D-F, between the labeled RNA species and its homologous unlabeled RNA was standardized at 100% competition, i.e. no labeled hybrid remaining. The relative quantity of labeled RNA remaining is taken as the relative difference between the RNA species. The black bars represent the L-specific RNA differences and the white bars represent the H-specific RNA differences. The labeled RNA species used in each experiment, e.g. Log*, was set at zero percent difference.

Panels A, B and C of Figure 32 represent hybridization-competition experiments (RNA/DNA = 10:1) with RNAs isolated from strain A26u⁻ sporulating in the SM-R media. It is evident from Figure 32A that

at stage T_2 of sporulation, cells do not transcribe a large number of vegetative genes from both of the DNA strands and that by T_4 , some of these vegetative genes were again transcribed. In addition, at T_2 new transcripts absent from both vegetative and sporulating T_4 cells were synthesized. New transcripts absent from other developing stages were also found in sporulating cells at stage T_4 . The differences between vegetative transcripts and the transcripts isolated from sporulating cells were much larger than those reported by Sumida-Yasumoto and Doi (1974). These investigators used the 2xSG sporulation medium which does not require the shift-down transfer performed with the SM-R sporulation media presented here and performed their competitions experiments at a RNA/DNA ratio of 100:1. Some of the large differences observed at the earlier sporulation stage, T_2 , may have resulted from such a radical change in the medium used, e.g. SM to R.

The differences between vegetative and stationary phase transcripts of cells grown in 121B medium are presented in Figure 32, panels D, E. and F. The "turn-off" of vegetative transcripts and the "turn-on" of new stationary phase transcripts was significant in light of the similar changes in RNA transcripts observed in sporulating cells (Sumida-Yasumoto and Doi, 1974; and Figure 32, panels A, B and C). It is demonstrated here that transcripts were synthesized in strain A26u⁻ during stationary phase in 121B medium which were absent from vegetative cells. The relative differences between vegetative and stationary phase cell transcripts were not

as large as those which occurred between vegetative and sporulating cell RNAs (compare panels B and E of Figure 32) although differences were observed. In addition, the portion of the vegetative genome not transcribed during stationary phase was larger in the sporulating cells than it was in the SpoM_c cells (compare panels A and D of Figure 32). The quantity of vegetative transcripts absent in T₂ cells was similar for both DNA strands during sporulation, e.g. approximately 40% whereas during stationary phase in 121B medium, a larger "turn-off" occurred from the L strand of DNA (see Figure 32 A and D).

In conclusion, a lack of vegetative gene transcription and a "turn-on" of stationary phase cistrons occurred in cells during sporulation in SM-R media or stationary phase in 121B medium. Sporulating cells appear to depress a larger portion of the genome transcribed during vegetative growth than the non-sporulating SpoM_c cells. In addition, chloramphenicol did not induce the transcription of a greater number of vegetative cistrons usually not transcribed during T₂ of stationary phase cells which was expected if the action of this drug was to restore vegetative sigma subunit activity (Segall et al., 1974; Pero et al., 1975).

DNA SYNTHESIS IN STATIONARY PHASE CULTURES OF BACILLUS SUBTILIS AND THE INITIATION OF SPORULATION

It was discussed in the Introduction that the completed spore has a full genetic complement which is present in one linkage group. The involvement of DNA synthesis in the initiation of sporulation, as discussed by Mandelstam et al. (1971) suggests that replication

must come to a halt as the cells in a population will be initiated into the scheme of sporulation. It was therefore interesting to analyze the patterns of DNA synthesis in sporulating (sp^+) and non-sporulating (sp^- and $SpoM_c$) cells. Bacillus subtilis cells were grown in the different sporulation media used previously, and samples of the growing and stationary phase cultures were assayed for the DNA content by the diphenylamine reaction. The results of these experiments are presented in Table 17. It is evident from these results that sporulating cells do not continue new rounds of DNA synthesis during stationary phase and that the total increase in the cellular DNA content after T_0 was less than 56%. Non-sporulating cells on the other hand, reinitiated new rounds of DNA synthesis at least once more after T_0 and showed an increase of greater than 50% in their DNA content. B. subtilis strain A26u⁻, an $SpoM_c$ phenocopy in 121B medium, behaved differently with respect to its DNA synthesis, when compared to the other $SpoM_c$ phenocopies. A26u⁻ cells in 121B medium contained 50% more DNA during stationary phase than was found in the same cells sporulating in SM-R and 2xSG media (see Table 17). W23 and BD29 cells demonstrated a much larger increase in the DNA synthesized during stationary phase in 121B medium. In respect to the quantities of DNA synthesized during stationary phase, $SpoM_c$ cell lines are very similar to the sp^- cell lines studied. However, when B. subtilis strain BD29 was grown in the SM-R sporulation media, new rounds of DNA replication were prevented. Strains A26u⁻ and BD29 have been shown previously to differ with respect to the rates

of RNA synthesis in 121B medium (see Figure 13, panels A and B). In 121B medium, A26u⁻ repeatedly did not demonstrate an RNA rate transition during the first hour of stationary phase but did stop new rounds of DNA synthesis, and BD29 which demonstrated the rate transition, did not prevent chromosome replication. In view of these results, it seemed that the decreased rates of RNA synthesis during the transition period and the prevention of new rounds of DNA synthesis during stationary phase were uncoupled in these strains of Bacillus subtilis.

A26u⁻ and BD29. In conclusion, the decreased RNA synthetic rates and the halt in DNA synthesis are both related to the sporulation events which occur during stationary phase, however it is unlikely that these two processes are preconditions for each other since they can be uncoupled in the SpoM_C strains presented here.

CONCLUDING REMARKS

Stationary phase presumably results from the depletion of some essential nutrient(s) from the growth medium by vegetative cells. Szulmajster and Hanson (1965) and Kawamura and Ito (1975) have demonstrated that as the glucose present in a culture medium is used up, the cells enter stationary phase. In this respect, stationary phase of sporulating and non-sporulating cell cultures share many characteristics, such as (1) a halt in the net accumulation of bulk cellular RNA, (2) a rapid decrease in the rates of RNA synthesis evident by T_1 , (3) a 50% reduction in the quantity of rRNA synthesized, and (4) increases in the quantity of mRNAs synthesized which hybridize to MAK-fractionated L and H DNA strands. Actively growing cell cultures, when deprived of glucose, also demonstrate a decrease in the overall rate of RNA synthesis and the depressed levels of rRNA synthesis until they have adjusted to the new carbon source. Unlike stationary phase cells however, glucose shift-down cultures, after adjusting to the new carbon source, increase the overall rate of RNA synthesis and the quantity of rRNA transcribed. These parameters in stationary phase cultures do not increase suggesting that although the depletion of glucose may have caused the original decreases in the rates of RNA synthesis and rRNA synthesis, some other factor (?) maintains the low synthetic rates. Since these low rates are observed in sp^+ , sp^- and $SpoM_c$ cells, they can not represent sporulation-specific template changes which occur

by T_2 . The "turn-off" of ribosomal RNA synthesis originally reported by Hussey et al. (1971) should more correctly be referred to as a slow down in the synthesis of rRNA since it represents only a 50% decrease in the quantities found in vegetative cells. The same decrease is observed in all three cell types studied and therefore does not solely constitute a sporulation specific event, even though it occurs during the first few hours of development, unless the phenomenon being studied occurs prior to the mutational or medium-induced blocks. Pero et al. (1975) have recently reported only a 75% depression of rRNA synthesis at T_2 of stationary phase in 121B medium.

Segall and Losick (1975) have demonstrated in an asporogenous mutant of B. subtilis, which retains the vegetative sigma subunit activity at T_2 of stationary phase, that σ_e transcription is 2.6 times more active than that found in the parent sporulating cell. These investigators however, still demonstrated that the overall rates of T_2 cellular RNA transcription and σ_e -specific transcription were depressed in both sp+ and sp- strains to 9-18% of the vegetative rates. The low rates of synthesis in the sp- mutant, suggests that the retention of the sigma subunit of RNA polymerase is not sufficient to maintain the vegetative rates of σ_e or total RNA synthesis. Segall et al. (1974) and Pero et al. (1975) have also demonstrated that treatment of sporulating cells with chloramphenicol restores the vegetative sigma subunit activity and increases the quantity of rRNA synthesized at T_2 of stationary phase to 70-75% of the levels observed in the vegetative

cells. Similar results have been observed in the studies presented here. The addition of chloramphenicol (CM) to cells at T_2 of stationary phase was observed to completely restore the vegetative levels of rRNA synthesis in sp⁻ cells but only partially raise these levels to 65% of the vegetative quantity, in sp⁺ and SpoM_C cells at the same period. In addition, CM was shown to increase the quantity and not the quality (e.g. new types) of L-strand specific mRNAs detectable by DNA-RNA hybridization only in sp⁺ and SpoM_C cells, whereas the quantity in sp⁻ cells remained unchanged. This suggests that sporulating cells, or cells genetically capable of sporulating, have acquired a new control factor(s) by T_2 which prevents a return to the vegetative levels of rRNA synthesis, whereas the spo0 (sp⁻) cells unable to proceed through sporulation do not acquire this characteristic. This difference between sp⁺ and sp⁻ cells may represent the stationary phase synthesis of a new transcription factor, which is not synthesized in cells blocked at stage T_0 . Table 18 demonstrates that both sp⁺ and sp⁻ strains of Bacillus subtilis increase the quantity of L-mRNA synthesized as the cells moved from vegetative growth (Log) to stationary phase (T_2). The increase in the quantities of T_2 L-mRNA in both the presence and absence of CM however, differed in these two cell types. Asporogenous cells synthesized a greater quantity (4.9%) of L-mRNA at T_2 than did sp⁺ cells (3.8%). In addition, the amount of hybridizable L-mRNA in sp⁺ cells in the presence of CM at T_2 was observed to increase from 3.8% to 5.1%

(see Table 18) whereas the relative quantity of L - m RNA in drug treated asporogenous cells at T_2 remained unchanged (see Legend to Table 18). These results strengthen the concept that stationary phase sp^- strains do not contain an inhibitor of quantitative L-mRNA transcription and therefore synthesize high levels (4.9%) of L-mRNA transcripts which can only be achieved in sp^+ cells (5.1%) when the action or synthesis of this inhibitor is blocked by the drug chloramphenicol. DNA-RNA hybridization and competition experiments demonstrated that the L-specific mRNA transcripts synthesized in the presence or absence of CM were identical. This result shows that any increase in the stationary phase cell content of L-mRNA in the presence of the drug is not due to different transcripts but rather a quantitative increase in the same species. DNA-RNA hybridization and competition experiments demonstrated that the L-specific mRNA transcripts synthesized in the presence and the absence of the drug were essentially identical, again demonstrating that the increases in RNA populations transcribed at T_2 in sp^+ cells.

The preincubation of sp^+ and $Spom_c$ cells with CM did not restore the vegetative levels of rRNA in cells at T_2 . Some other process(es), which occurs prior to T_2 must be responsible for maintaining the depressed levels of rRNA synthesized in sp^+ cells. The events described in this study which occur prior to T_2 are summarized for sp^+ , sp^- and $Spom_c$ cells in Table 19. As shown, the depressed levels of rRNA synthesis and the quantitative increases in L and H mRNA species are not specific to spore development. The transition in the rate

of RNA synthesis however, appears to be related to the sporulation sequence, since cells of strain A26u⁻ in 121B medium which do not decrease the rate of RNA synthesis are also blocked before the production of serine protease activity at T₀ and heat resistant spores at T₈. The production of alkaline phosphatase activity, which precedes heat resistance (S/V) is the sporulation-specific event which appeared at T₂ of development. A most interesting result presented in this table is the uncoupling of the regulation of DNA replication and the rate of RNA synthesis (compare A26u⁻ and BD29 in 121B medium). Apparently these two processes, which develop in sporulating cells, are not prerequisites for each other and occur independently. The effect of chloramphenicol on the transcription of stationary phase cell at T₂ was surprising. It suggests the presence of two different factors affecting transcription (1) a CM-sensitive inhibitor which partially depresses L-mRNA and rRNA transcription in sp⁺ cells and is completely responsible for the depressed levels of rRNA synthesis in sp⁻ cells and (2) a CM-insensitive event which maintains the depressed levels of rRNA synthesis and is not reversed by the addition of the drug. The former example may represent the action of the magic spot nucleotides (MSI and MSII) which, as discussed in the introduction, appear during the transition period of stationary phase, e.g. T_{1/2}. The latter example may represent a new CM-stable transcription factor capable of directing the synthesis of stationary phase transcripts which

have been demonstrated to exist.

A scheme for the events occurring during the first two hours of stationary phase is presented in Figure 33. The events studied here are those which appear to be common to a specific period during stationary phase, referred to as the transition period, T_0 to T_1 . The production of two highly phosphorylated nucleotides, HPN III and IV, which appear by T_1 of sporulation, has been implicated in the initiation of sporulation-specific development (Rhaese et. al., 1976). These nucleotides, which appear in response to carbon and/or phosphate depletion in the medium, seem to coincide with the commitment to sporulation with respect to glucose, ie. by T_1 . The study of Kawamura and Ito (1975), in addition to the results presented here, suggests that there are different transcriptional alterations occurring in sporulating cells during the first few hours in stationary phase. These investigators have shown that the bacteriophage $\phi 29$ transcription is depressed between T_1 and T_2 to only 21% of the levels found during vegetative growth. ϕe on the other hand still maintained 42% of the vegetative transcription activity. In addition, the multiplication of ϕe was not blocked in these sporulating cells until T_3 , whereas a five-fold decrease in the phage $\phi 29$ multiplication was detected by T_1 . These results suggest that RNA synthesis and DNA replication in $\phi 29$ closely parallel the molecular changes evident in sporulating cells during the transition period, whereas ϕe represents the changes occurring in the developing cell nearer the time of initiation of the sporulation sequence (see Figure 33).

Figure 33 demonstrates the scheme of events as they occur during the transition and initiation periods of sporulation. Changes in the growth medium, caused by the growing cells, e.g. nutrient depletion, lead to changes in the overall metabolism. Some of these stationary phase events are specific to the developing system (events marked with asterisks) while the others are common to all B. subtilis cells during stationary phase. These events occurring before the initiation period, T_1 , are reversible and the cells appear to behave similarly whether they are sp⁺ or sp⁻.

This study has demonstrated that the overall RNA synthetic patterns of sporulating and non-sporulating cells are very similar, although differences do exist in the quantities and types of RNAs synthesized. The control over the synthesis of nucleic acids during the transition period, eventually determines the path the growing cell will encounter, e.g. sporulation or stationary-phase maintenance. The events leading to this play a very important role in the fate of the developing cell. Below are listed some of the areas that might be studied to identify some of the mechanisms involved in the control of the initiation of development during the transition period.

- (1) Depressed rates of RNA synthesis. Since phosphorylated nucleotides i.e. ppGpp, pppGpp, ppApp, pppApp, pppAppp and ppXpUp, have been demonstrated to increase in vivo during the first few hours of sporulation, it would be interesting to identify their role in the regulation of RNA synthesis during this period. This could be demonstrated in vitro by the quantity and type of transcripts synthesized in the presence and absence of these nucleotides. The in vitro transcripts could then be compared to the in vivo transcripts. In addition, these nucleotides could be tested in vitro to see if they qualify as the "inhibitor" of vegetative

sigma factor to prevent the binding of sigma to the core polymerase during the stationary phase.

- (2) Are the phosphorylated nucleotides capable of stimulating L-DNA specific transcription since the increase of these compounds corresponds to slower growth rates and sporulation?
- (3) Are the transcripts synthesized during stationary phase and sporulation different in their translational efficiencies from the vegetative transcripts? Since both vegetative and sporulation-specific or related transcripts are synthesized during the stationary phase, sporulation may proceed by the preferred translation of a specific class of mRNA species.
- (4) What is the nature of the metabolic block which allows a sp^+ phenotype to mimic a sp^- cell type? Strain A26u⁻, which does not undergo a rate transition during the first hour of stationary phase seems the likely candidate for study. Possibly the "inhibitor" of vegetative sigma subunit activity is blocked from synthesis or from performing its function, whatever that may be.

To date, many laboratories have studied the time period in which B. subtilis cells demonstrate changes in their overall metabolism, which in some manner, induce sporulation-specific processes, i.e. T₀-T₂. Very few reports however, have identified the metabolic events reported here, which are common to all stationary phase cells and which appear to precede

the initiation of most sporulation-specific events.

APPENDIX A

I. Growth MediaA. Spizizen Minimal Salts MediumFinal Concentration

Basal Salts:

KH_2PO_4	4.0 mM
K_2HPO_4	80.0 mM
Sodium Citrate	3.4 mM
MgSO_4	1.0 mM

Supplements added separately after autoclaving:

Glucose	0.5 %
Sodium Glutamate	1.0 %
Casein Hydrolysate	0.5 %

B. Veal Infusion Broth

Veal Infusion (Difco)	12.5 g
Yeast Extract (Difco)	5.0 g

Add to 1 liter of water and autoclave.

II. Sporulation Media

A. Schaeffer Modified Nutrient Sporulation Medium (2xSG)

Nutrient Broth (Difco)	16.0 g
KCl	27.0 mM
MgSO_4	2.0 mM

Add to 1 liter of water and autoclave.

Supplements added separately:

1. after autoclaving

MnCl ₂	0.1 mM
Ca (NO ₃) ₂	0.1 mM
Glucose	0.1 %

2. after sterile filtration

Fe SO ₄	1.0 x 10 ⁻³ mM
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B. Sonenshein and Roscoe Sporulation Medium (1218)

Basal Salts:

NaCl	80.0 mM
KCl	20.0 mM
NH ₄ Cl	20.0 mM
ZnCl ₂	2.0 x 10 ⁻³ mM
Na ₂ SO ₄	25.0 mM
TRIS (Tris-hydroxymethyl aminomethane)	120.0 mM
Casein Hydrolysate	0.2 %

Add water to 1 liter and autoclave.

Supplements added separately:

1. after autoclaving

MgCl ₂	1.0 mM
CaCl ₂	0.2 mM
MnCl ₂	0.1 mM
K ₂ HPO ₄	2.5 mM
Sodium Citrate	3.4 mM
Glucose	0.2 %
Tryptophan	0.1 mM

2. Adjust pH to 7.5 with 6N HCl containing FeCl_3 ,
to give a final concentration of 0.15 mM
 FeCl_3 .

C. Sterlini and Mandelstam Growth (SM) and Sporulation (R) Media

1. Growth Medium (SM)

Casein Hydrolysate	0.86%
D,L-alanine	240.0 mM
L-Asparagine	8.0 mM
Mn SO_4	0.1 mM
Mg SO_4	0.4 mM
NH_4Cl	10.0 mM
Na_2SO_4	0.75 mM
NH_4NO_3	1.2 mM
CaCl_2	0.1 mM

Add water to 1 liter, adjust pH to 7.1 with KOH
and autoclave.

Supplements added separately:

a. after autoclaving:

KH_2PO_4	10.0 mM
L-glutamate, sodium salt	20.0 mM
L-tryptophan	0.1 mM
Glucose	0.2%

b. sterile filtered

FeCl_3	1.0×10^{-3} mM
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2. Sporulation Resuspension Medium (R)

MgSO_4	40.0 mM
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Add water to 1 liter, adjust pH to 7.1 with KOH and autoclave.

Supplements added separately:

a. after auto claving

KH_2PO_4	0.5 mM
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NH_4Cl	10.0 mM
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Na_2SO_4	0.75 mM
--------------------------	---------

NH_4NO_3	1.2 mM
--------------------------	--------

MnCl_2	0.1 mM
-----------------	--------

CaCl_2	0.2 mM
-----------------	--------

b. Sterile filtered

FeCl_3	4.0×10^{-4} mM
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III. Tryptose Blood Agar Base (TBAB)

TBAB (Difco)	33.0 g
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Bacto agar	5.0 g
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add to 1 liter of water and autoclave.

IV. Dilution Saline

NaCl	8.7 g
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K_2HPO_4	3.5 g
--------------------------	-------

add water to 1 liter, adjust pH to 7.5 and autoclave.

V Germination Medium (GM)

A. Spizizen Minimal Salts Medium

(see Appendix A, IA)

B. Supplements added separately after autoclaving:

	<u>Final Concentration</u>
Glucose	0.5%
Uridine	25.0 $\mu\text{g/ml}$
L-alanine	100.0 $\mu\text{g/ml}$
L-argininal	20.0 $\mu\text{g/ml}$
L-asparagine	50.0 $\mu\text{g/ml}$
L-glutamate	100.0 $\mu\text{g/ml}$
L-histidine	50.0 $\mu\text{g/ml}$
L-isoleucine	10.0 $\mu\text{g/ml}$
L-leucine	5.0 $\mu\text{g/ml}$
L-methionine	5.0 $\mu\text{g/ml}$
L-serine	5.0 $\mu\text{g/ml}$
L-threonine	20.0 $\mu\text{g/ml}$
L-valine	20.0 $\mu\text{g/ml}$

APPENDIX B

Correlation Coefficient*:

The determination of the statistical correlation (r) between the two sets of measures, X, (generation time; u) and Y (ribosomal RNA content), was calculated from the equation:

$$r = \frac{n \sum XY - (\sum X)(\sum Y)}{\sqrt{(n \sum X^2 - (\sum X)^2)(n \sum Y^2 - (\sum Y)^2)}}$$

where n = number of individual experiments.

*Taken from: Statistical Methods: A problem-solving approach
by V. Gourevitch, publisher Allyn and Bacon, Inc.
Boston, 1965.

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Table 1

The *Bacillus subtilis* Strains Used in This Study

<u>Strain</u> *	<u>Genotype</u> ^a	<u>Spore Genotype Designation</u> ^b	<u>Spore Phenotype</u> ^c	<u>Source</u>
A26u ⁻	ura ⁻	sp ⁺	wild type	D. Dubnau
BD29	arg ⁻ leu ⁻	sp ⁺	wild type	D. Dubnau
W23	S ^r E ^r M ^r	sp ⁺	Pr ⁺ Ab ⁺ Cp ⁻	D. Dubnau
NCTC 3610 (ATCC 6051)	wild type	sp ⁺	Pr ⁺ Ab ⁺ Cp ⁻	D. Dean
SP98-8	leu ⁻ thr ⁻ met ⁻	spo0 _a 30	Pr ⁻ Ab ⁻ Cp ⁻	I. Takahashi
SPC14-1	cys A	spo0 ₇	Pr ^{+/-} Ab ⁺ Cp ⁺	I. Takahashi
OA5NA	wild type	spo0 _a 47	Pr ⁻ Ab ⁻ Cp ⁻	R. Losick
rfm 3Y	rfm	rfm	Pr ⁻ Ab ^{+/-} Cp ⁻	R. Losick

*

The only isogenic strains are NCTC 3610 and rfm 3Y.

^aura, arg, leu, thr, met and cys are auxothrophic requirements for uracil, arginine, leucine, threonine, methionine and cysteine respectively. Rfm, S, E and M are the notations for resistances to the drugs rifampicin, streptomycin, erythromycin and mitomycin respectively.

^bThese spore notations refer to the mapped positions of these spore mutations on the *B.subtilis* chromosomal map (Young and Wilson, 1972).

^cThe phenotype of the individual spo0 mutants is presented as discussed in the article by Young and Wilson (1972). Ab, Pr, and Cp refer to antibiotic production, protease production and competence for transformation; +, - and +/- refer to the presence, absence and low levels of the respective activities presented.

Table 2

*The actual sporulation frequency (S/V) is expressed as the ratio of the number of viable cells at T_{24} before (V) and after (S) heating a sample of the cell culture at 80° for 10 minutes, ie. $(S/V) \times 100$.

**The corrected sporulation frequency is expressed as the ratio of the number of viable cells earlier in stationary phase, e.g. T_2 , (V) and the number of heat resistant cells (S) after heating a T_{24} sample of the cell culture for 10 minutes at 80° .

***Ratio of alkaline phosphatase units present in 5 ml of cell culture assayed at T_1 and T_6 , ie. T_6/T_1 . See Materials and Methods for the assay.

Table 2

Sporulation-Specific Events as They Occur in the Sterlini and Mandelstam Sporulation Media (SM-R)

Stage	Stage (T _n = hours)	Cell Number		S/V(%) Actual*	S/V (%) Corrected**	Alkaline Phosphatase T ₆ /T ₁ ***
		Viability (x10 ⁸)	Heat Resistant Cells			
3610 sp ⁺	T ₂₄	5.80	5.60 x 10 ⁸	96.6	-	-
A26u ⁻ sp ⁺	T ₂	1.46				
	T ₂₄	1.48	1.42 x 10 ⁸	95.9	97.2	10.2
rfm 3Y sp ⁻	T ₂	4.62				
	T ₂₄	0.82	2.00 x 10 ⁷	24.4	4.3	-
SPC14-1 sp ⁻	T ₂	1.45				
	T ₂₄	0.06	1.00 x 10 ³	0.017	0.0007	0.9
SP98-8 sp ⁻	T ₂	1.69				
	T ₂₄	0.05	1.40 x 10 ⁵	2.8	0.083	1.3
OASNA sp ⁻	T ₂	1.81				
	T ₂₄	0.14	4.00 x 10 ²	0.003	0.0002	1.5

Table 3

Sporulation-Specific Events as They Occur in the Sonenshein and Roscoe Sporulation Medium (121B)

Stage	Stage (T _n = hours)	Cell Number		S/V (%) Actual [*]	S/V (%) Corrected ^{**}	Alkaline Phosphatase T ₆ /T ₁ ^{***}
		Viability (x10 ⁸)	Heat Resistant Cells			
3610 sp ⁺	T ₂₄ ^a	10.35	9.85 x 10 ⁸	95.2	-	-
3610 sp ⁺	T ₂₄	14.05	8.95 x 10 ⁸	63.7	-	6.8
A26u ⁻ sp ⁺	T ₂	15.25				
	T ₂₄	3.68	1.70 x 10 ⁸	46.2	11.1	1.1
BD29 sp ⁺	T ₈	9.10				
	T ₂₄	6.25	4.00 x 10 ⁷	6.4	4.4	1.0
W23 sp ⁺	T ₁₂	7.75				
	T ₂₄	3.90	2.00 x 10 ⁷	5.1	2.6	0.02
rfm 3Y sp ⁻	T ₂	26.10				
	T ₂₄	5.90	2.00 x 10 ⁵	0.034	0.0044	-
SPC14-1 sp ⁻	T ₂	11.00				
	T ₂₄	4.15	3.00 x 10 ³	0.0007	0.0003	-

SP98-8 sp ⁻	T ₂	19.60				
	T ₂₄	8.20	1.00 x 10 ⁶	0.12	0.051	-

*Actual S/V (%), determined as described in Table 2.

**Corrected S/V (%), determined as described in Table 2.

***Alkaline phosphatase, ratio of the units present at T₁ and T₆; see Materilas and Methods for assay.

^a121B medium containing 0.1mM Magnesium ion concentration. All others presented in this table contain 1.0 mM Magnesium.

Table 4

Rates of RNA Synthesis During the Transition Period in the Different Sporulation Media

<u>Strain</u>	<u>Medium</u>	Capacity to <u>Sporulate</u>	Rates of RNA Synthesis [*] Relative to T ₀	
			<u>T₁ (%)</u>	<u>T₅ (%)</u>
A26u ⁻	2xSG	+	27	7
A26u ⁻	SM-R	+	2	9
A26u ⁻	Spizizen	-	88	-
A26u ⁻	121B ^a	-	100	53
A26u ⁻	121B ^b	-	100	56
3610	121B ^b	+	5	3
W23	121B ^b	-	2	29
BD29	121B ^b	-	4	1
SP98-8	121B ^b	-	1	4
rfm 3Y	121B ^b	-	4	7 ^c

* Data taken from Figures 8 through 14.

^a Magnesium concentration in 121B medium is 0.1 mM.

^b Magnesium concentration in 121B medium is 1.0 mM.

^c T₄ instead of T₅.

Table 5

Hybridization-competition behavior of vegetative stable-RNA of *Bacillus subtilis*.

Expt. No.	<u>DNA</u> Prep.No.	Strand	Input Ratio DNA/RNA	Unlabeled srRNA Competitor ^a	cpm Complexed After RNase ^b	³ H-RNA Hybridized (%)	DNA (H) or (L) Hybridized (%)
1	I	H	50:1	--	1,650	36.6	0.73
		H	50:1	50 x (V)	18	0.4	
2	I	H	75:1	--	2,215	49.1	0.67
		H	75:1	50 x (V)	45	1.0	
3	II	H	200:1	--	4,490	99.7	0.50
		H	200:1	100 x (V)	43	1.0	
		H	200:1	100 x (S ₁)	167	3.7	
4	II	L	95:1	--	2	0.04	0.0005

^aUnlabeled messenger-free srRNA from vegetative (V) cells was extracted after treatment with rifampicin (30 µg/ml) for 20 minutes. Similarly, unlabeled RNA from sporulating cells (S₁) at stage T_{2.5} was extracted after treatment with rifampicin (100 µg/ml) for 40 minutes.

^bEach resction mixture contained 0.17 µg ³H-srRNA (specific activity, 26,500 cpm/µg), MAK fractionated H or L strands, 6xSSC in a tota volume of 1.60, 1.28, 0.68 and 3.0 ml for experiments No. 1,2,3, and 4 respective. The reactions were incubated at 67° for 18 hours and treated as described.

Table 6

The Quantitative Hybridization of Vegetative and Sporulation RNAs to Individual DNA Fractions Eluted From a MAK Column

Strand	MAK Fraction ^a	DNA Content in MAK Fraction (%) ^b	RNA Hybridized (%) ^d			
			Vegetative Total	mRNA ^c	Sporulation T ₂ Total	mRNA
L	A	9.0	-	2.6	-	3.5
	B	12.7	-	2.4	-	4.2
	C	9.7	-	2.6	-	4.5
	D	14.0	-	2.6	-	5.5
	E	4.6	-	5.6	-	7.9
H	F	2.4	12.1	3.1	15.4	9.7
	G	8.3	30.2	7.7	27.0	17.0
	H	11.1	66.4	16.9	40.4	25.4
	I	9.5	88.6	22.5	55.7	35.0
	J	12.8	92.5	23.5	52.1	32.7
	K	5.9	88.0	22.4	42.7	26.8

^aThese are the same fractions as shown in Figure 22.

^bThe individually pooled DNA fractions, A-E and F-K, represent portions of the respective L or H fractions of the MAK profile presented in Figure 22.

^cThe DNA/RNA ratio was 75:1.

^dThe quantity of rRNA present in the labeled RNA preparations hybridized with the DNA, as determined by competition-hybridization experiments with purified rRNA, was found to be 74.6% and 37.2% for vegetative and sporulation T₂ RNAs respectively. The proportion of mRNA in the labeled RNA preparation was determined from the calculation: Total RNA (%) - rRNA (%) = mRNA (%). This determination yields a minimum estimate of the total quantities of L and H specific mRNAs.

Table 7

^aAll double labeled RNA preparations used here were purified from peak I material by MAK chromatography as described previously.

^bLog(3) and Log(8) are vegetative cells grown in minimal salts medium (Spizizen, 1958) or SM medium and pulse-labeled with(³H)Uridine (44 or 17 μ c /ml) for a period of 3 and 8 minutes respectively. In experiments 3,4,5 and 6 the same amount of(³H)Uridine was used (10 μ c /ml) and the amount of(³²P)phosphate as μ c /ml, was 33 for experiment 3, 65 for T₂ and 70 for T₄ in experiment 4, and 50 for experiments 5 and 6.

^cThe various resction mixtures contained the following amounts of RNA in μ g: expts. 1 and 2, 0.06 (spec.act. 55,200) and 0.35 (spec.act. 4,630); expt. 3, 2.87 (spec.act. 7,270 and 476 for ³H and ³²P respectively), 0.83 (spec.act. 6,500 and 1,955 for ³H and ³²P respectively), 1.68 (spec.act. 7,160 and 900 for ³H and ³²P respectively) for T₁, T_{2.5} and T₅; expt. 4, 0.5 (spec.act. 5,130 and 2,162 for ³H and ³²P respectively), 0.5 (spec. act. 4,400 and 5,110 for ³H and ³²P respectively) for T₂ and T₄; expt. 5, (spec.act. 500,200 and 120,450 for ³H and ³²P respectively); expt. 6, (spec.act. 381,000 and 9,065 for ³H and ³²P respectively). The reaction mixtures contained the appropriate amounts of MAK-fractionated H-DNA strands, 6xSSC in a total volume of 0.68 ml for experiments 1,2 and 4, 3.0 ml for experiment 3, and 1.66 ml for experiments 5 and 6. The reactions were incubated for 18 hours at 68° as described in the Materials and Methods. V, S_I and S_{II} are srRNA preparations extracted from cells during vegetative growth, stage T_{2.5} and stage T₆ of sporulation respectively.

Table 7 Hybridization-Competition Behavior of RNA
Growth and with ^{32}P -phosphate During Various

Strain	Expt. No.	Stage of Pulse Labeling ^b	H-DNA/RNA Input Ratio	Unlabeled Competitor srRNA ^c
A26u ⁻	1	Log(3)	200:1 200:1 200:1	- 100 x V 100 x S _I
A26u ⁻	2	Log(8)	200:1 200:1 200:1	- 100 x V 100 x S _I
A26u ⁻	3	T ₁	75:1 75:1	- 50 x V
		T _{2.5}	75:1 75:1	- 50 x V
		T ₅	75:1 75:1	- 50 x V
A26u ⁻	4	T ₂	200:1 200:1 200:1	- 100 x V 100 x S _I
		T ₄	200:1 200:1 200:1	- 100 x V 100 x S _I
OA5NA	5	T _{2.5}	200:1 200:1	- 100 x V
			200:1 200:1 200:1	- 100 x S _I 100 x S _{II}
SP98-8	6	T _{2.5}	200:1 200:1	- 100 x V
			200:1 200:1 200:1	- 100 x S _I 100 x S _{II}

Preparations^a Labeled with ³H-Uridine During Vegetative
Sporulation Stages of *Bacillus subtilis*

Complexed after RNase		RNA Hybridized (%)		srRNA in Labeled Preparation (%)	
³ H	³² P	³ H	³² P	³ H	³² P
2,497	-	75.4	-	-	-
1,422	-	43.0	-	43.0	-
1,425	-	43.0	-	43.0	-
1,588	-	98.0	-	-	-
599	-	35.2	-	64.1	-
524	-	33.0	-	66.4	-
20,887	1,190	99.9	87.0	-	-
565	856	2.8	62.5	97.2	28.2
5,360	890	99.9	55.2	-	-
84	325	1.8	20.2	98.2	63.4
8,441	637	70.2	42.1	-	-
109	183	0.9	12.1	98.3	72.3
2,546	1,006	99.9	93.1	-	-
56	337	2.2	31.2	97.8	66.5
43	341	1.7	31.5	98.3	66.2
2,497	1,742	98.7	68.2	-	-
50	407	2.2	15.9	97.8	76.7
40	377	1.9	14.8	98.1	78.3
80,019	1,939	99.9	10.1	-	-
1,012	512	1.3	2.7	98.2	73.6
74,809	1,780	94.3	9.0	-	-
607	378	0.8	2.0	99.2	78.7
126	630	0.2	3.5	99.8	64.6
134,490	1,592	99.9	49.8	-	-
1,509	445	1.1	13.9	98.9	72.1
119,708	1,392	89.0	34.9	-	-
1,081	403	0.8	10.1	99.1	71.0
297	359	0.2	9.0	99.7	74.2

Table 8

^aThis value of S/V is corrected for the loss in viability at T₂₄ (see Table 2).

^bsrRNA is composed of total stable RNA isolated from Bacillus subtilis strain A26u⁻ after Actinomycin D treatment (10 µg/ml for 10 minutes).

^cReaction mixtures contained the following amounts of three minute pulse-labeled(³H)RNA in a H-DNA/RNA ratio of 150:1, expt.1: Log, 0.34 ug (spec.act. 8,097), T₁ 0.32 µg (spec.act. 7,308), T₂, 0.52 µg (spec. act. 3,166); expt. 2, Log, 0.1 µg (spec.act. 154,120), T₂, 0.1 µg (spec.act. 23,990); expt. 3, Log, 0.21 µg (spec.act. 8,296), T₁, 0.52 µg (spec. act. 7,552), T₂, 0.45 µg (spec.act. 14,760). Total volumes for expts. 1,2 and 3 were 4.58, 1.0 and 4.58 ml respectively.

Table 8

Synthesis of Stable (srRNA) RNA by *Bacillus subtilis* Strain A26u⁻ in the Different Sporulation Media

Expt. No.	Medium & S/V (%) ^a	Stage	100 X srRNA ^b (+/-)	RNA Hybridized to H-DNA after RNase ^c		srRNA Content (%)
				(cpm)	(%)	
1	2xSG (80.2)	Log	-	2,031	74.7	84.7
			+	311	11.4	
		T ₁	-	1,764	76.2	65.9
			+	602	26.0	
		T ₂	-	1,018	61.8	51.7
			+	492	29.9	
2	SM-R (97.8)	Log	-	12,952	79.8	76.4
			+	3,064	18.9	
		T ₂	-	1,320	55.0	38.7
			+	809	33.7	
3	121B (11.0)	Log	-	1,209	70.7	88.5
			+	140	8.2	
		T ₁	-	1,613	41.1	56.7
			+	699	17.8	
		T ₂	-	2,541	38.4	46.0
			+	1,374	20.8	

Table 9

^aThis S/V determination is corrected for the loss in viability at T₂₄ (see Table 3).

^bReaction mixtures contained the following amounts of three minute pulse-labeled(³H)RNA in a L-DNA/RNA ratio of 100:1, expt. 1, Log, 0.6 μ g (spec.act. 9,043), T₂, 0.3 μ g (spec.act. 14,085); expt. 2, Log, 0.3 μ g (spec.act. 162,545), T₂, 0.3 μ g (spec.act. 29,255); expt. 3, Log, 0.3 μ g (spec.act. 235,350), T₂, 1.0 μ g (spec.act. 16,330). Total volumes for experiments 1,2 and 3 were 3.8 ml.

^crRNA is composed of just the 5S, 16S and 23S unlabeled rRNAs isolated from vegetative B. subtilis A26u⁻ ribosomes (see Figure 18).

^dReaction mixtures contained the following amounts of three minute pulse-labeled(³H)RNA in a H-DNA/RNA ratio of 150:1, expt. 1, Log, 0.2 μ g, T₂, 0.1 μ g; expt. 2, Log, 0.1 μ g, T₂, 0.3 μ g; expt. 3, Log, 0.1 μ g, T₂, 0.4 μ g. Total volumes for experiments 1,2 and 3 were 1.0, 1.0 and 2.1 ml respectively.

Table 9

RNA Synthesis of *Bacillus subtilis* sp⁺ Strains in the Sonenshein and Roscoe Sporulation Medium (121B)

Expt. No.	Strain & S/V (%) ^a	Stage	RNA Hybridized to L-DNA after RNase ^b		100 X rRNA ^c (+/-)	RNA Hybridized to H-DNA after RNase ^d		rRNA Content (%)
			(cpm)	(%)		(cpm)	(%)	
1	3610 (63.7)	Log	52	1.9	-	1,252	69.2	
					+	181	10.0	85.6
		T ₂	236	5.6	-	1,018	72.3	
					+	472	33.5	53.6
2	W23 (2.6)	Log	953	2.0	-	11,148	68.6	
					+	1,049	6.5	90.6
		T ₂	171	2.0	-	1,140	39.0	
					+	313	10.7	72.6
3	BD29 (4.4)	Log	416	2.6	-	19,752	83.9	
					+	2,408	1.7	87.8
		T ₂	130	2.7	-	729	44.6	
					+	553	33.8	24.2

Table 10

^aThis S/V determination is corrected for the loss in viability at T₂₄ (see Table 3).

^bReaction mixtures contained the following amounts of three minute pulse-labeled (³H)RNA in a L-DNA/RNA ratio of 100:1, expt. 1, Log, 0.1 μg (spec. act. 424,620), T₂, 0.1 μg (spec.act. 28,105); expt. 2, Log, 0.1 μg (spec. act. 480,015), T₂, 0.1 μg (spec.act. 39,605). Total volumes for both experiments were 1.1 ml.

^crRNA is the ribosomal RNA isolated from ribosomes described in the legend to Table 9.

^dReaction mixtures contained the following amounts of three minute pulse-labeled (³H)RNA in a H-DNA/RNA ratio of 150:1, expt. 1, 0.1 μg for both Log and T₂; expt. 2, 0.1 μg for both Log and T₂. Total volumes for both experiments were 1.1 ml.

Table 10

RNA Synthesis of *Bacillus subtilis* sp⁻ Strains in the Sonenshein and Roscoe Sporulation Medium (121B)

Expt. No.	Strain & S/V (%) ^a	Stage	RNA Hybridized L-DNA after RNase ^b		100 X rRNA ^c (+/-)	RNA Hybridized H-DNA after RNase ^d		rRNA Content (%)
			(cpm)	(%)		(cpm)	(%)	
1	SP98-8 (5.1x10 ⁻²)	Log	1,290	3.0	-	30,159	71.4	51.7
					+	14,574	34.5	
		T ₂	140	5.0	-	1,676	59.6	24.2
					+	1,270	45.2	
2	rfm 3Y (4.4x10 ⁻³)	Log	1,533	3.2	-	45,196	94.2	65.6
					+	15,543	31.0	
		T ₂	309	7.8	-	2,961	74.7	33.8
					+	1,959	49.4	

Table 11

^aThis S/V determination is corrected for the loss of viability at T₂₄ (see Table 3).

^brRNA is the ribosomal RNA isolated from ribosomes described in the legend to Table 9.

^cReaction mixtures contained the following amounts of three minute pulse-labeled (³H) rRNA in a H-DNA/RNA ratio of 150:1, expt. 1, Log, 0.21 μ g (spec. act. 8,296), T₂, 0.45 μ g (spec.act. 14,760); expt. 2, Log 0.20 μ g (spec. act. 7,802), T₂, 0.1 μ g (spec. act. 18,490); expt. 3, Log, 0.2 μ g (spec. act. 9,043), T₂, 0.1 μ g (spec.act. 14,085); expt. 4, Log 0.1 μ g (spec. act. 10,490), T₂, 0.1 μ g (spec.act. 12,870). Total volumes of the experiments 1,2,3 and 4 were 4.6, 1.0, 1.0 and 1.0 respectively.

Table 11

The Effect of Magnesium Concentration of the Synthesis of Ribosomal RNA by *Bacillus subtilis* Growing in the Sonenshein and Roscoe Sporulation Medium (121B)

Expt. No.	Strain & S/V (%) ^a	Magnesium (mM)	Stage	100 X rRNA ^b (+/-)	RNA Hybridized to H-DNA after RNase ^c		rRNA Content (%)
					(cpm)	(%)	
1	A26u ⁻ (11.1)	1.0	Log	- +	1,209 140	70.7 8.2	88.5
			T ₂	- +	2,541 1,374	38.4 20.8	46.0
2	A26u ⁻ (9.2)	0.1	Log	- +	1,298 298	83.2 19.1	87.1
			T ₂	- +	1,526 1,023	82.5 55.3	33.0
3	3610 (63.7)	1.0	Log	- +	1,252 181	69.2 10.0	85.6
			T ₂	- +	1,018 472	72.3 33.5	53.6
4	3610 (70.2)	0.1	Log	- +	938 216	89.4 20.6	77.0
			T ₂	- +	802 506	62.3 39.3	37.0

Table 12

^aReaction mixtures contained the following amounts of three minute pulse-labeled (³H)RNA in a L-DNA/RNA ratio of 100:1, expt.1, Log, 0.3 μ g (spec.act. 397,340); expt. 2A, LAG, not done, (spec.act. 558), LAG-CM, 2.0 μ g (spec. act. 499); expt. 2B, Log, 0.3 μ g (spec.act. 40,120), Log-CM, 0.1 μ g (spec. act. 38,905). Total volumes for experiments 1, 2A and 2B were 1.9 ml.

^brRNA is the ribosomal RNA isolated from ribosomes described in the legend to Table 9.

^cReaction mixtures contained the following amounts of three minutes pulse-labeled(³H)RNA in a H-DNA/RNA ratio of 150:1, expt. 1, Log, 0.1 μ g; expt. 2A, LAG, 2.0 μ g, LAG-CM, 2.0 μ g; expt. 2B, Log, 0.1 μ g, Log-CM, 0.1 μ g. Total volumes for experiments 1, 2A and 2B were 1.2, 10.4 (LAG), 6.6 (LAG-CM), 1.2 (Log) and 1.0 (Log-CM) respectively.

^dLAG refers to the adjustment time of the cells to a new carbon source (see Figure 8B).

^eCells pretreated with chloramphenicol (200 μ g/ml for 20 minutes) prior to pulse labeling with ³H Uridine.

Table 12

Artificially Induced Carbon-Source Shift-Down in Vegetative Cells of *Bacillus subtilis* Strain A26u⁻ and the Effect on the Rate of Ribosomal RNA Synthesis

Expt. No.	Stage	Modification in Medium	Generations per hour (u)	RNA Hybridized to L-DNA after RNase ^a		100 X rRNA ^b (+/-)	RNA Hybridized to H-DNA after RNase ^c		rRNA Content (%)
				(cpm)	(%)		(cpm)	(%)	
1	Log	none	1.23	7,246	6.1	-	32,834	82.6	46.9
						+	17,466	43.9	
2A	LAG ^d	-glucose	0	-	-	-	867	77.7	9.0
						+	789	70.7	
	LAG-CM ^e	-glucose	0	79	7.9	-	933	93.5	62.5
						+	350	35.1	
2B	Log	-glucose	0.24	966	8.0	-	3,395	84.6	40.6
						+	2,023	50.4	
	Log-CM ^e	-glucose	r	82	2.1	-	2,394	61.5	64.0
						+	862	22.1	

Table 13

^aThe S/V values presented for A26u⁻ are corrected for the loss in viability at T₂₄ (see Tables 2 and 3). The S/V values presented for 3610 are the actual values measured at T₂₄.

^bChloramphenicol (CM)-treated cells (200 µg/ml for 20 minutes prior to labeling).

^cReaction mixtures contained the following amounts of three minute pulse-labeled (³H)RNA in a L-DNA/RNA ratio of 100:1, expt.1, Log (154,120 cpm/µg) 0.3, Log-CM (120,405 cpm/µg) 0.1, T₂ (23,990 cpm/µg) 0.3, T₂-CM (102,910 cpm/ug) 0.1 µg; expt. 2, Log (20,560 cpm/µg) 0.3, Log-CM (145,355 cpm/µg) 0.1, T₂ (67,205 cpm/µg) 0.3, T₂-CM (77,365 cpm/µg) 0.3 µg; expt. 3, Log (13,525 cpm/µg) 0.3, Log-CM (61,980 cpm/µg) 0.1, T₂ (25,410 cpm/µg) 0.3, T₂-CM (72,670 cpm/µg) 0.1 µg; expt.4, Log (9,043 cpm/µg) 0.6, T₂ (14,085 cpm/µg) 0.3, T₂-CM (15,070 cpm/µg) 0.3 µg. Total volumes for experiments 1,2,3 and 4 were 0.92, 0.92 3.80 and 3.80 ml respectively.

^dRibosomal RNA extracted from ribosomes described in the legend to Table 9.

^eReaction mixtures contained 0.1 µg of (³H)RNA, in a H-DNA/RNA ratio of 150:1 for all experiments above except for expt. 4, which used 0.2 µg RNA. The total volume for each reaction mixture in experiments 1 through 4, was 1.0 ml-

Table 13

The Effect of Chloramphenicol on the Synthesis of RNA During Stationary Phase of Sporulating and Non-Sporulating Cells of *Bacillus subtilis*

Strain	Expt. No.	Medium & S/V (%) ^a	Stage ^b	RNA Hybridized L-DNA after RNase ^c		100 X rRNA ^d (+/-)	RNA Hybridized H-DNA after RNase ^e		rRNA Content (%)
				(cpm)	(%)		(cpm)	(%)	
A26u ⁻	1	SM-R (97.2)	Log	1,101	2.4	-	12,952	84.0	76.4
						+	3,064	19.9	
			Log-CM	607	5.1	-	11,452	85.1	
						+	3,296	27.4	
			T ₂	207	3.8	-	1,320	55.0	
						+	890	33.7	
	2	121B (11.1)	Log	103	1.7	-	1,543	75.1	76.1
						+	369	18.0	
			Log-CM	483	3.3	-	13,331	91.7	
						+	3,245	22.3	
			T ₂	601	3.1	-	4,335	64.5	
						+	2,714	40.4	

3610	3	SM-R (96.6)	T ₂ -CM	1,608	6.9	-	4,919	63.6	48.4
						+	2,540	32.8	
			Log	91	2.2	-	1,237	93.3	
						+	363	27.4	
			Log-CM	278	4.5	-	4,323	69.7	
						+	1,481	23.9	
	4	121B (95.2)	T ₂	213	2.8	-	1,362	53.6	32.7
						+	917	36.1	
			T ₂ -CM	262	3.6	-	4,473	61.6	
						+	2,020	27.8	
			Log	52	1.9	-	1,252	69.2	
						+	181	10.0	
			T ₂	236	5.6	-	1,018	72.3	53.6
						+	472	33.5	
			T ₂ -CM	237	5.2	-	991	65.8	
						+	407	27.0	

Table 14

^aThese S/V values are corrected for the loss in viability at T₂₄ (see Tables 2 and 3).

^bChloramphenicol(CM)-treated cells (200 µg/ml for 20 minutes prior to labeling).

^cReaction mixtures contained 0.1 µg of(³H)RNA in all experiments except for expt. 3, Log-CM (0.3) and T₂ (0.4) in a L-DNA/RNA ratio of 100:1. The specific activities of the RNA preparations, as cpm/ug, were: expt.1, Log-CM (276,150), T₂ (35,230), T₂-CM (85,110); expt.2, Log (424,620), T₂ (28,105), T₂-CM (306,405); expt.3, Log-CM (10,748), T₂ (6,155), T₂-CM (75,620); expt.4, Log (480,015), T₂(39,605), T₂-CM (195,450). Total volumes were 1.1 ml for experiments 1,2 and 3, and 2.3 for expt. 3.

^dRibosomal RNA prepared from ribosomes as described in the legend to Table 9.

^eReaction mixtures contained 0.1 µg of(³H)RNA in a H-DNA/RNA ratio of 150:1, except expt. 4 which contained the following amounts of RNA: Log-CM (0.3 µg) and T₂ (0.4 µg). Total volumes were 1.0 ml for expt.1, 1.1 ml for expts. 2 and 4, and 4.5 ml for expt. 3.

Table 14

The Effect of Chloramphenicol on the Synthesis of RNA During Stationary Phase of Asporogenous Cells of *Bacillus subtilis*

Mutant Strain	Expt. No.	Medium & S/V (%) ^a	Stage ^b	RNA Hybridized L-DNA after RNase ^c		100 X rRNA ^d (+/-)	RNA Hybridized H-DNA after RNase ^e		rRNA Content (%)
				(cpm)	(%)		(cpm)	(%)	
SP98-8	1	SM-R (0.083)	Log-CM	579	2.1	-	26,430	95.7	61.7
						+	10,128	36.7	
			T ₂	81	2.3	-	1,366	38.8	
						+	736	20.9	
			T ₂ -CM	125	1.5	-	4,500	52.9	
						+	1,107	13.0	
	2	121B (0.051)	Log	1,290	3.0	-	30,159	71.4	51.7
						+	14,574	34.5	
			T ₂	140	5.0	-	1,676	59.6	
						+	1,270	45.2	
			T ₂ -CM	1,102	3.6	-	21,370	69.7	
						+	10,678	34.8	

rfm 3Y	3	SM-R (4.3)	Log-CM	75	2.3	-	2,192	68.0	
						+	333	10.3	
			T ₂	112	4.6	-	610	24.8	
						+	388	15.8	
			T ₂ -CM	89	1.2	-	1,878	24.8	
						+	364	4.8	
	4	121B (0.004)	Log	1,533	3.2	-	45,196	94.2	
						+	15,543	32.4	
			T ₂	309	7.8	-	2,961	74.7	
						+	1,959	49.4	
			T ₂ -CM	1,096	5.6	-	18,478	94.5	
						+	8,834	45.2	

Table 15

Relative Quantities of mRNA, tRNA and rRNA in *Bacillus subtilis* strain A26u⁻ During Growth, Sporulation and Stationary-Phase

Medium	Stage	Stable RNA Content (%) ^a			Messenger RNA Content (%)		H/L Hybridization Ratio ^c
		tRNA ^b	rRNA	Total	H	L	
SM-R ^d	Log	-	-	74.6	23.0	2.4	9.6
	T ₂	-	-	37.2	59.0	3.8	15.5
	T ₂ +CM	-	-	47.8	47.6	4.6	10.3
121B ^e	Log	2.2	80.5	82.7	14.9	2.4	6.3
	T ₂	3.5	46.8	50.3	45.3	4.2	10.8
	T ₂ +CM	2.2	56.9	59.1	36.3	4.7	7.7
2xSG ^f	Log	-	-	83.4	15.5	1.1	14.1
	T ₂	-	-	50.3	47.0	2.7	17.4

^aDetermined from a competition-hybridization experiment at a DNA/RNA ratio of 150:1 and in the presence of 100 fold excess unlabeled srRNA.

^bCalculated from the difference between the quantity of hybrid remaining in the presence of excess unlabeled srRNA and rRNA preparations.

^cA quantitative value determined at high DNA/RNA ratios.

^dValues determined from the results presented in Table 13, expt. 1.

^eValues determined from the same RNA preparations described in Figure 27.

^fValues determined from the results presented in Table 8, expt.1.

Table 16

^aThe stable RNA (srRNA) content was determined by a separate competition experiment using DNA/RNA ratios of 100:1 to 200:1.

^bAll hybridizations were performed at near saturation levels of RNA (RNA/DNA = 100:1). The reaction mixtures, in a total volume of 0.08 to 0.3 ml, contained the appropriate amounts of MAK fractionated DNA and the following amounts of RNA in μ g: Expt.1, Log (spec. act. 21,000) 19; Expt.2, Log(3) (see Table 7; spec.act. 55,200) 16; Expt.3, Log(8) (see Table 7; spec.act. 4,630) 50, T_2 (spec.act. 5,130 and 1,886 for ^3H and ^{32}P respectively) 20, T_4 (spec.act. 4,400 and 3,087 for ^3H and ^{32}P respectively) 20; Expt.4, 60 μ g RNA for both Log (spec. act. 15,780) and T_2 (spec.act. 30,630).

^cDetermined from the calculation: (μ g of input mRNA bound in hybrid per μ g DNA) $\times$ 100. The proportion of mRNA in each RNA preparation was calculated as described in Table 6.

^dCells grown in Spizizen's minimal salts medium supplemented with amino acids to support germination of spores (GM, germination medium; see Appendix A) were labeled with (^3H) Uridine (24 and 44 μ c /ml for expts. 1 and 2 respectively) for 3 minutes.

^efrom Jeng and Doi (1974).

Table 16

Saturation of DNA by In Vivo Labeled RNA Isolated From Vegetative, Sporulating and Stationary-Phase Cells of *Bacillus subtilis* Strain A26u⁻

Expt. No.	Medium	Spore Phenotype	Labeled RNA Species	srRNA ^a Content (%)	RNA ^b Hybridized (%)		Assymetry Ratio H/L	DNA Transcribed (%) ^c	
					H	L		H	L
1	GM ^d	-	Log	31.5	2.55	0.77	3.3	3.83	1.16
2	GM ^d	-	Log(3)	43.0	1.46	0.30	5.0	2.78	0.56
3	SM-R	sp ⁺	Log(8)	65.2	2.28	0.52	4.4	4.42	1.00
			T ₂	66.3	2.73	0.61	3.9	8.08	2.08
			T ₄	77.5	1.74	0.42	4.1	7.74	1.87
4	121B	SpoM _C	Log	84.8	1.16	0.25	4.6	7.33	1.59
			T ₂	52.6	2.48	0.51	4.9	5.23	1.08
5	2xSG ^e (WB746)	sp ⁺	Log	-	-	-	-	(7.0-10.3)	
			T ₂	-	-	-	-	(8.5-12.1)	

Table 17

Regulation of DNA Synthesis During Stationary-Phase of Sporulating
and Non-sporulating *Bacillus subtilis* Cells

Strain	Medium	Capacity to Sporulate	DNA Synthesis ^a		Heat Resistance (Corrected S/V) ^b (%)
			Stage Completed (T _n = hours)	Increase from T ₀ (%)	
A26u ⁻	2xSG	+	T ₄	53	80.0
A26u ⁻	SM-R	+	T ₂	50 $\pm 3^c$	96.0
BD29	SM-R	+	T ₅	56	76.0
3610	121B	+	T ₂	53	80.0
A26u ⁻	121B	-	T ₄	75	11.0
BD29	121B	-	T ₆	201	4.0
W23	121B	-	T ₆	287	3.0
SP98-8	SM-R	-	T ₄	108	0.0002
OA5NA	SM-R	-	T ₅	143	0.083

^aEstimated from the diphenylamine reaction (Burton, 1968) as modified by Giles and Meyers (1965).

^bThese S/V determinations were corrected for the loss of viability at T₂₄ as described in the legend to Table 2.

^cThe average of three independent experiments.

Table 18

The Effect of Chloramphenicol on L-Specific Messenger RNA Transcription
in sp^+ and sp^- Cells of *Bacillus subtilis*

<u>Genotype^a</u>	<u>Stage</u>	Quantity of mRNA Hybridized to L-DNA (%) ^b	
		<u>(-CM)</u>	<u>(+CM)</u>
<u>sp^+</u>	Log	2.1 $\pm$ 0.3	4.3 $\pm$ 0.8
	T ₂	3.8 $\pm$ 0.9	5.1 $\pm$ 1.2
<u>sp^-</u>	Log	3.1 $\pm$ 0.1 ^c	2.2 $\pm$ 0.1 ^d
	T ₂	4.9 $\pm$ 1.9	3.0 $\pm$ 1.8

^aThe sp^+ and sp^- strains presented here are A26u⁻ and 3610, and SP98-8 and OB3Y respectively. The calculated relative quantities of L-mRNA synthesized at T₂ in the absence and presence of the drug (CM) were 6.8% and 10.9% for sp^+ strains and 7.5% and 7.8% for sp^- strains respectively.

^bThese results are the averages of the results presented in Tables 13 and 14, for each of the strains in both 121B and SM-R media.

^cSamples only from 121B medium.

^dSamples only from SM medium.

Table 19

Summary of the Changes Observed by T₂ of Stationary Phase in Various

Strain	Medium	Spore Phenotype	50 % Decrease rRNA	Quantitative Increase in	
				H-mRNA	L-mRNA
3610	SM-R	sp ⁺	+	+	+
	121B	sp ⁺	+	+	+
A26u ⁻	SM-R	sp ⁺	+	+	+
	2xSG	sp ⁺	+	+	+
	121B	SpoM _C	+	+	+
BD29 & W23	121B	SpoM _C	+	+	+
SP98-8 & rfm 3Y	SM-R	sp ⁻	+	+	+
	121B	sp ⁻	+	+	+

+, - and +/- refer to the presence, absence or variability of the
 ND, not done.

Strains of Bacillus subtilis in the Three Sporulation Media

<u>Rate of Synthesis</u>	<u>Ability to Reinitiate</u>		<u>Chloramphenicol-Induced</u>		<u>Alkaline Phosphatase</u>
	<u>New Rounds of DNA Synthesis</u>	<u>Increase L-mRNA</u>	<u>Vegetative Levels of rRNA</u>		
+	ND	+	-		+
+	+	+/-	+/-		+
+	+	+	-		+
+	+	ND	ND		+
-	+/-	+	-		-
+	-	ND	ND		-
+	-	-	+		-
+	-	-	+		-

events assayed.

Figure 1 Morphological and biochemical changes occurring during sporulation of bacilli.*

*Taken from Young and Wilson (1972)

Figure 1 Morphological and Biochemical Changes
Occuring During Sporulation of Bacilli

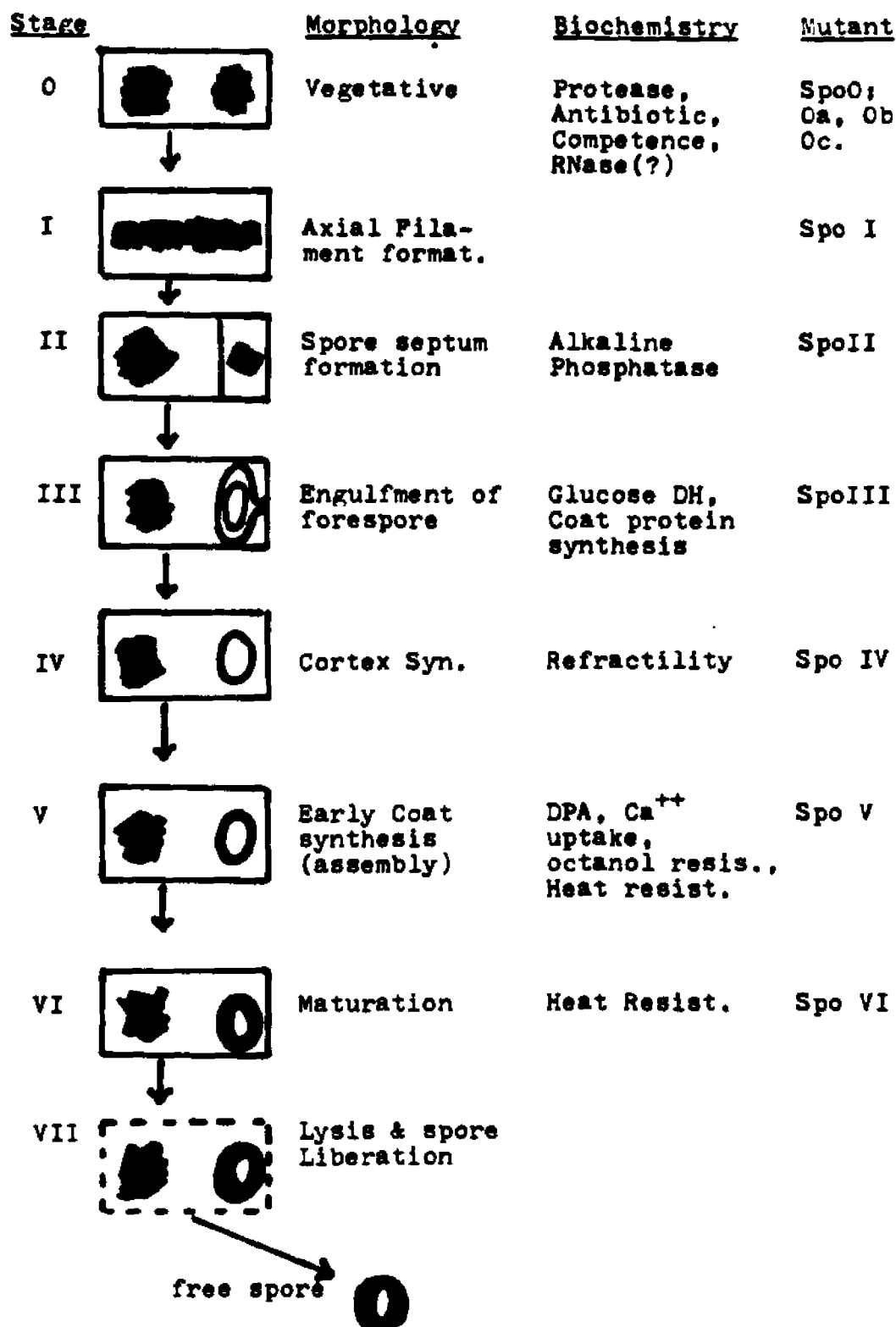


Figure 2 The Bacillus subtilis chromosomal map taken from Young and Wilson (1975). The markers identified on the outer periphery of the circular map have been identified as requirements for: adenine (pur A), cysteine (cys A and B), isoleucine (ilv A), arginine (arg A), leucine (leu), threonine (thr), methionine (met A,B,C and D) and pyrimidine (pyr). Cit B denotes the absence of a functional aconitase enzyme. Resistance to the drugs rifampicin and fusidic acid are noted as rfm and fus respectively. The two locations of the ribosomal and transfer RNA cistron loci are noted as rRNA A and B, and tRNA A and B. Sporulation mutations are mapped on the inner part of the circular map. Spo0, II and III refer to the biochemical or morphological block in sporulation as described in Figure 1. Spo 0_a, 0_b and 0_c refer to the spo0 mutants blocked at different developmental points before the formation of the axial filament as described in Figure 1 and Table 1. The arrows demonstrate the positions of the spo0 mutants used in these studies.

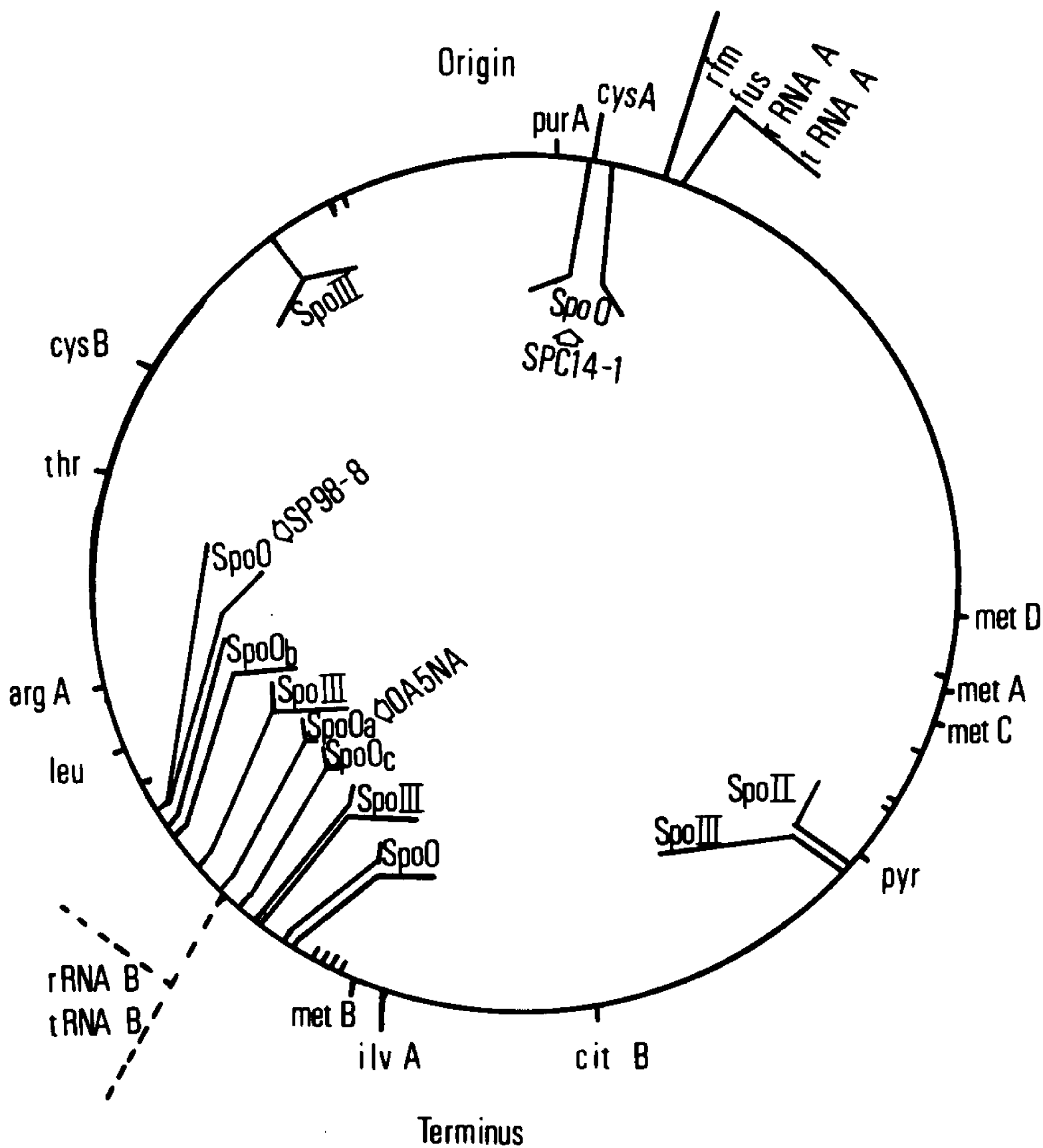


Figure 3 Growth and sporulation of Bacillus subtilis strain A26u⁻ in the Sterlini and Mandelstam Sporulation media (SM-R). Changes in bacterial mass (○) were monitored by a Klett-Summerson Colorimeter using a #66 filter. At a klett of 60 the cells were centrifuged, washed (dashed line) and resuspended in R medium. The time of resuspension is T₀. Panel A: Samples of the culture medium (40-50 ml) were centrifuged to pellet the cells. The pH of the extracellular medium was monitored with a pH meter (Leeds and Northrup) throughout growth and sporulation. Panel B: Throughout the experiment, duplicate samples (9.5 ml) were removed and precipitated with cold TCA (final concentration 5%) and fractionated according to the Schmidt-Thannhauser procedure (1945). Protein (●) was determined with the folin reagent as described by Lowry et al., (1951); RNA (▲) was determined by the orcinol reaction (Schneider, 1957); and DNA (△) was determined by the diphenylamine reaction (Burton, 1968; Giles and Meyers, 1965). Panel C: Alkaline phosphatase activity (□ ; 1 unit of activity = 1 nanomole of nitrophenol produced per minute at 37°) was estimated from duplicate 5 ml samples of cell culture according to Torriani (1960); Spores were estimated by viable counts before (V) and after heating (S) for 10 minutes at 80° and is expressed as the sporulation frequency (■ ; S/V x 100).

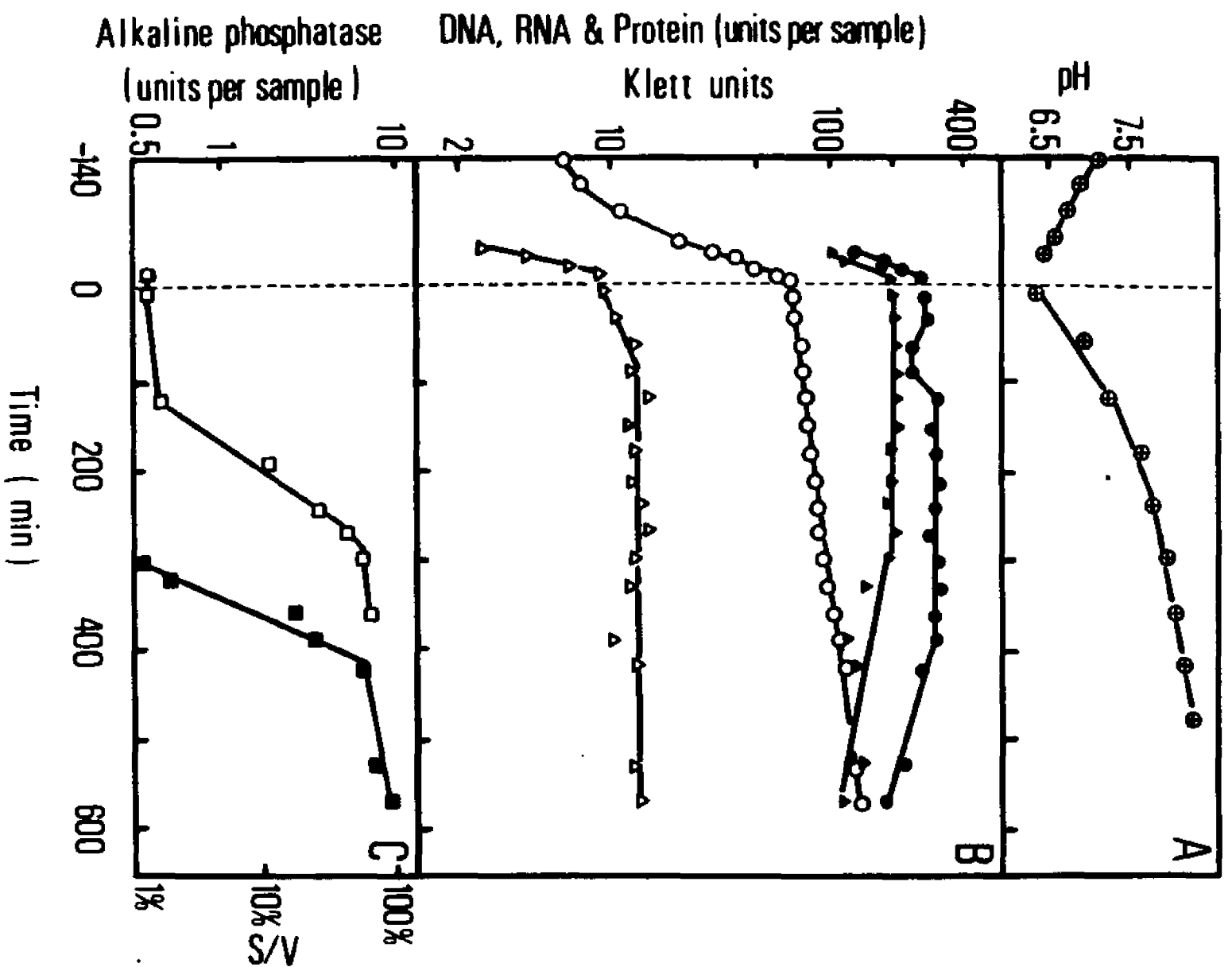


Figure 4 Growth and stationary phase patterns of asporogenous Bacillus subtilis strain SP98-8 in the Sterlini and Mandelstam Sporulation media (SM-R). At a klett of 74 the cells were centrifuged, washed (double-dashed line) and resuspended in R medium. The time of resuspension is T_0 . Changes in bacterial mass ($\circ$), DNA (Δ) and RNA ($\blacktriangle$) and Alkaline Phosphatase ($\square$) are as described in Figure 3.

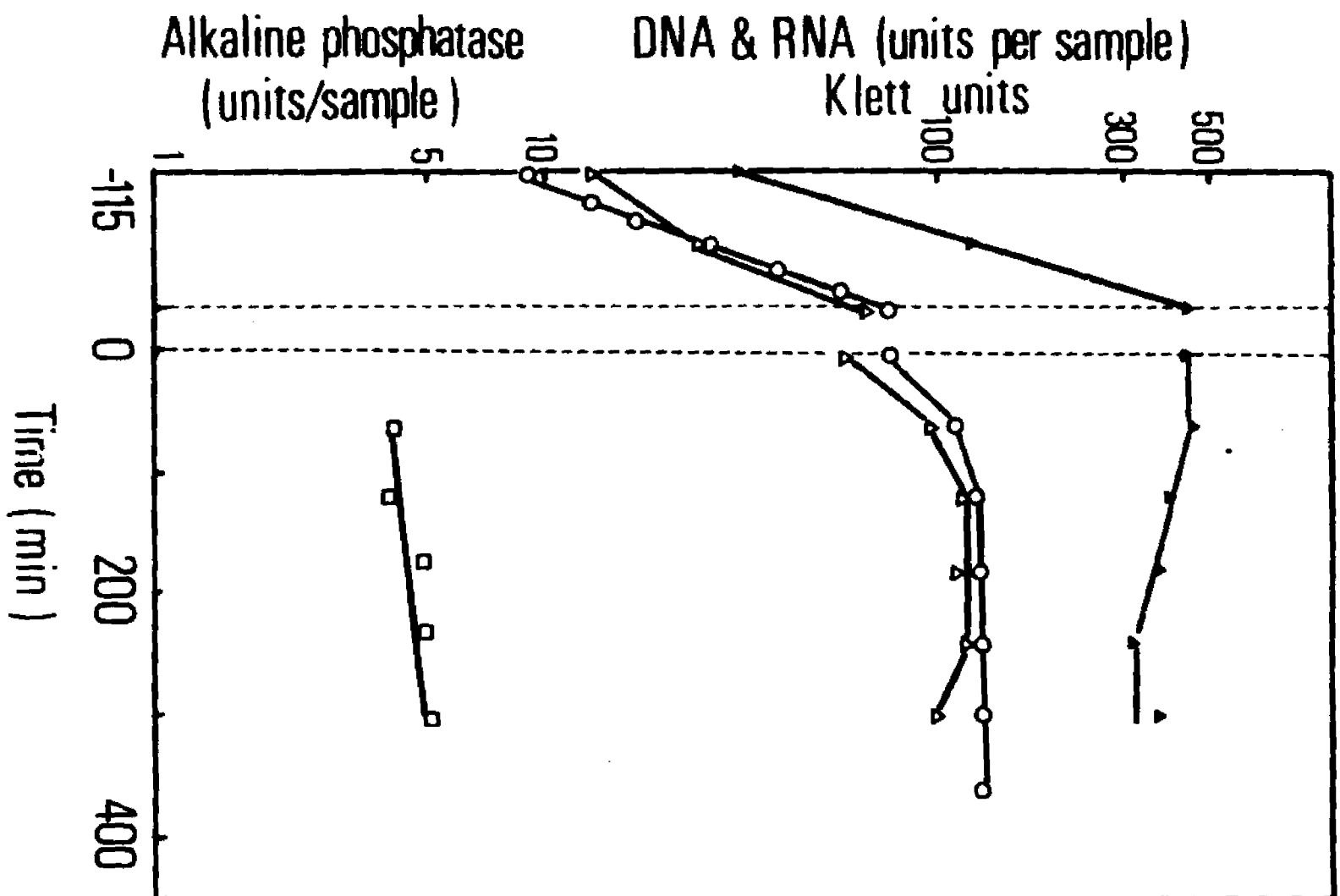


Figure 5 Growth and sporulation of Bacillus subtilis strain 3610 in the Sonenshein and Roscoe Sporulation medium (121B). B. subtilis cells were grown in 121B medium until stationary phase at which time they were centrifuged and resuspended at a klett of 55 in fresh 121B medium as shown. T_0 is indicated by the vertical dashed line and is the first observable change in the pattern of growth. Changes in bacterial mass ($\circ$) and estimations of DNA ($\triangle$), RNA ($\blacktriangle$), and Alkaline Phosphatase ($\square$) are as described in Figure 3.

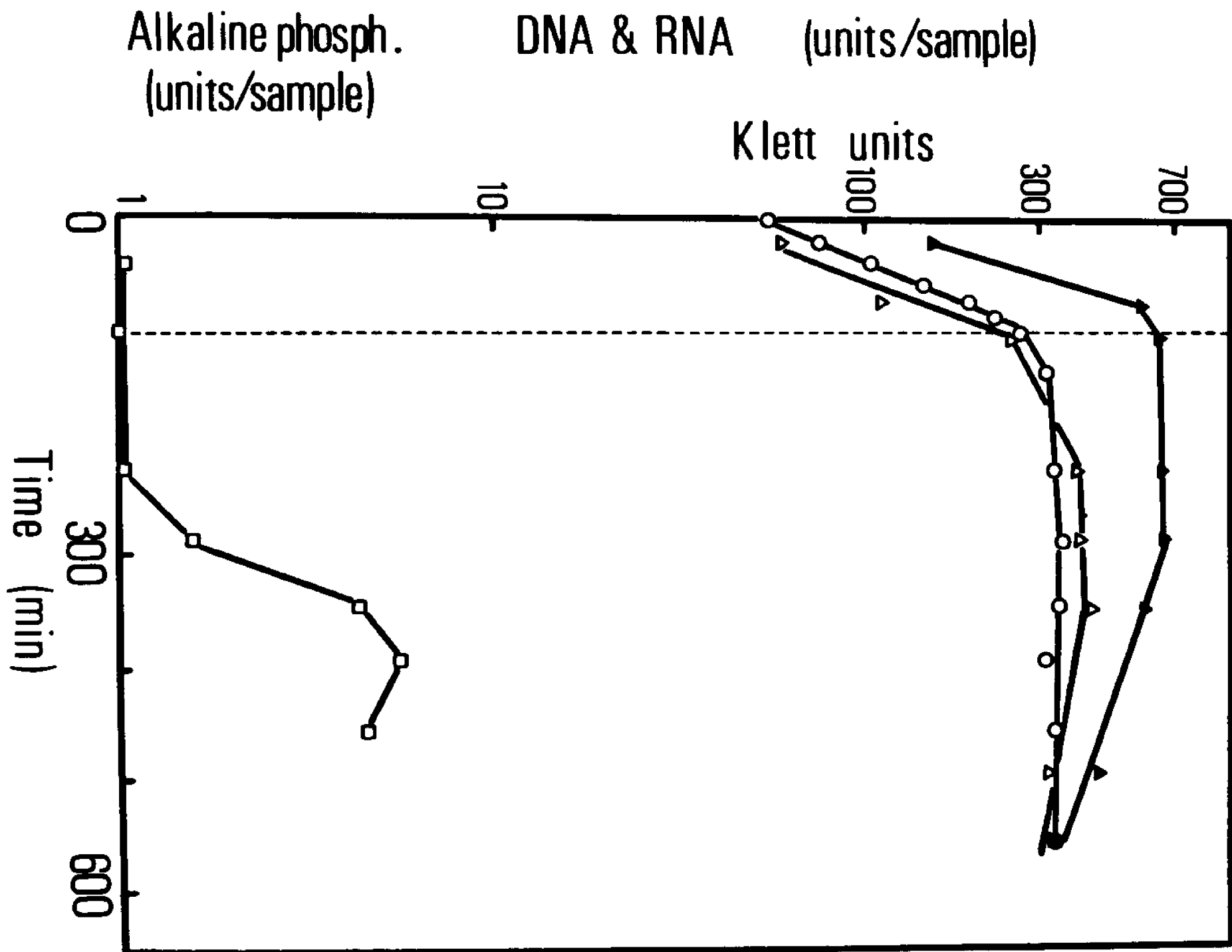


Figure 6 Growth and stationary phase patterns of Bacillus subtilis strain A26u⁻ in the Sonenshein and Roscoe Sporulation medium (121B). B. subtilis cells were grown in 121B medium until stationary phase, centrifuged and washed and resuspended at a klett of 65 in fresh 121B medium as shown. T_0 is indicated by the vertical dashed line and represents the first observable change in the logarithmic pattern of growth. Changes in bacterial mass ($\circ$), DNA (Δ), RNA ($\blacktriangle$) and Alkaline Phosphatase ($\square$) are described in Figure 3. The viable number of cells in the population ($\bullet$) was determined by making a serial dilution of the cell culture and plating duplicate samples on TBAB agar plates.

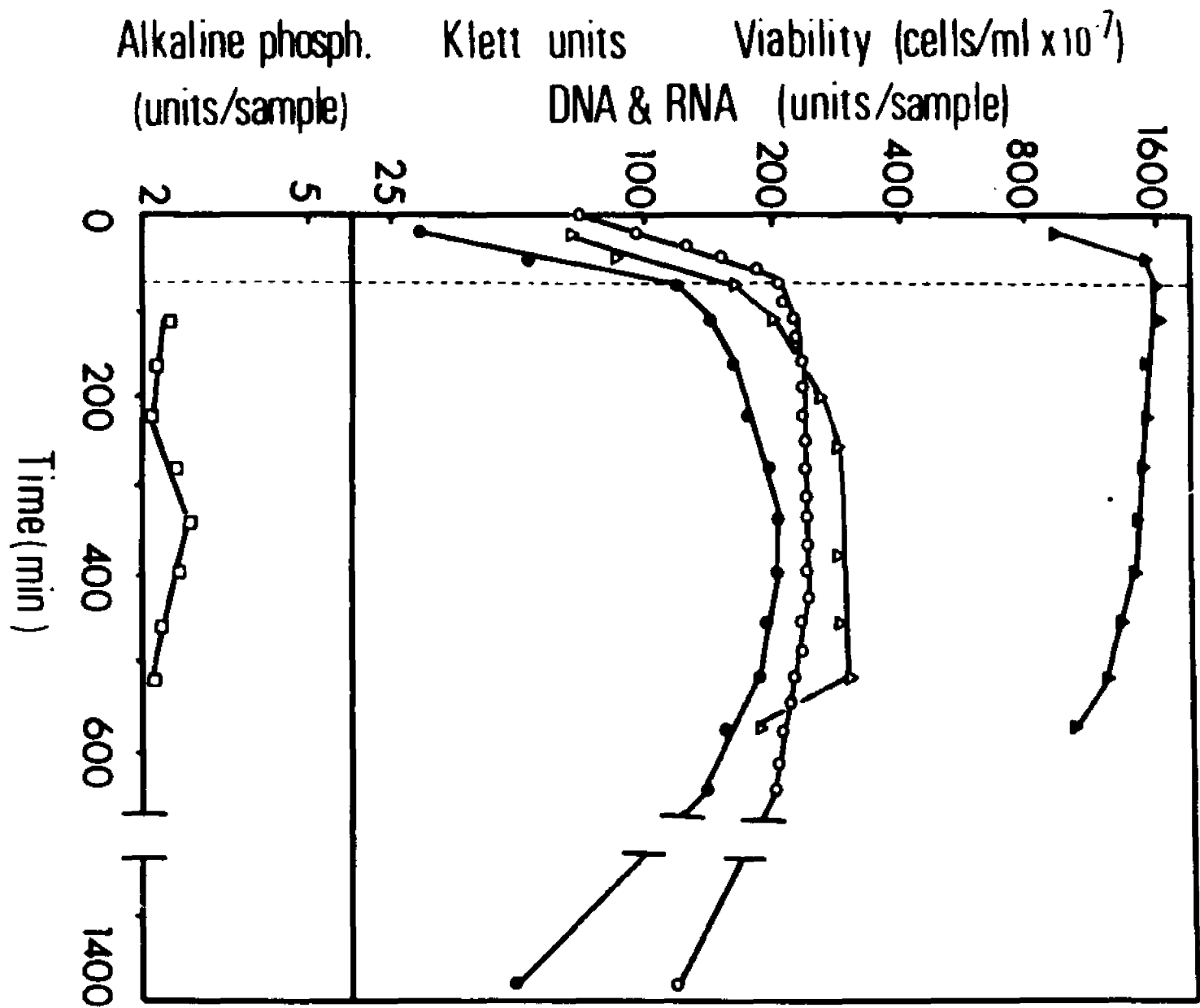


Figure 7 Production of serine protease during stationary phase in the Sonenshein and Roscoe Sporulation medium (121B). *Bacillus subtilis* strains 3610 and A26u⁻ were grown in the 121B medium until stationary phase as shown in figures 5 and 6. At different periods of growth and stationary phase cell culture samples were centrifuged to remove cells and the cell-free supernatant assayed for extracellular protease activity in the presence or absence of the serine protease inhibitor PMSF (final concentration 2mM; prepared fresh). Reaction mixtures contained 0.1-0.2 ml of cell-free supernatant from cell cultures at a similar Klett reading and 8-10,000 cpm of ¹⁴C-labeled *B. subtilis* protein, in a Tris magnesium buffer. The reaction tubes were incubated for 15 minutes at 37° and stopped with the addition of one volume of cold TCA (10%). The quantity of protein digested is a measure of the amount of protease activity present and is represented as the amount of digestion at different periods in stationary phase relative to that amount present in vegetative cell cultures (%). (A) 3610 protease activity with (▲) and without (○) PMSF, (B) Comparison of 3610 and A26u⁻ protease activities, (C) A26u⁻ protease activity with (▲) and without (●) PMSF, (D) A26u⁻ intracellular protease activity, from cell pellet, with (▲) and without (⊙).

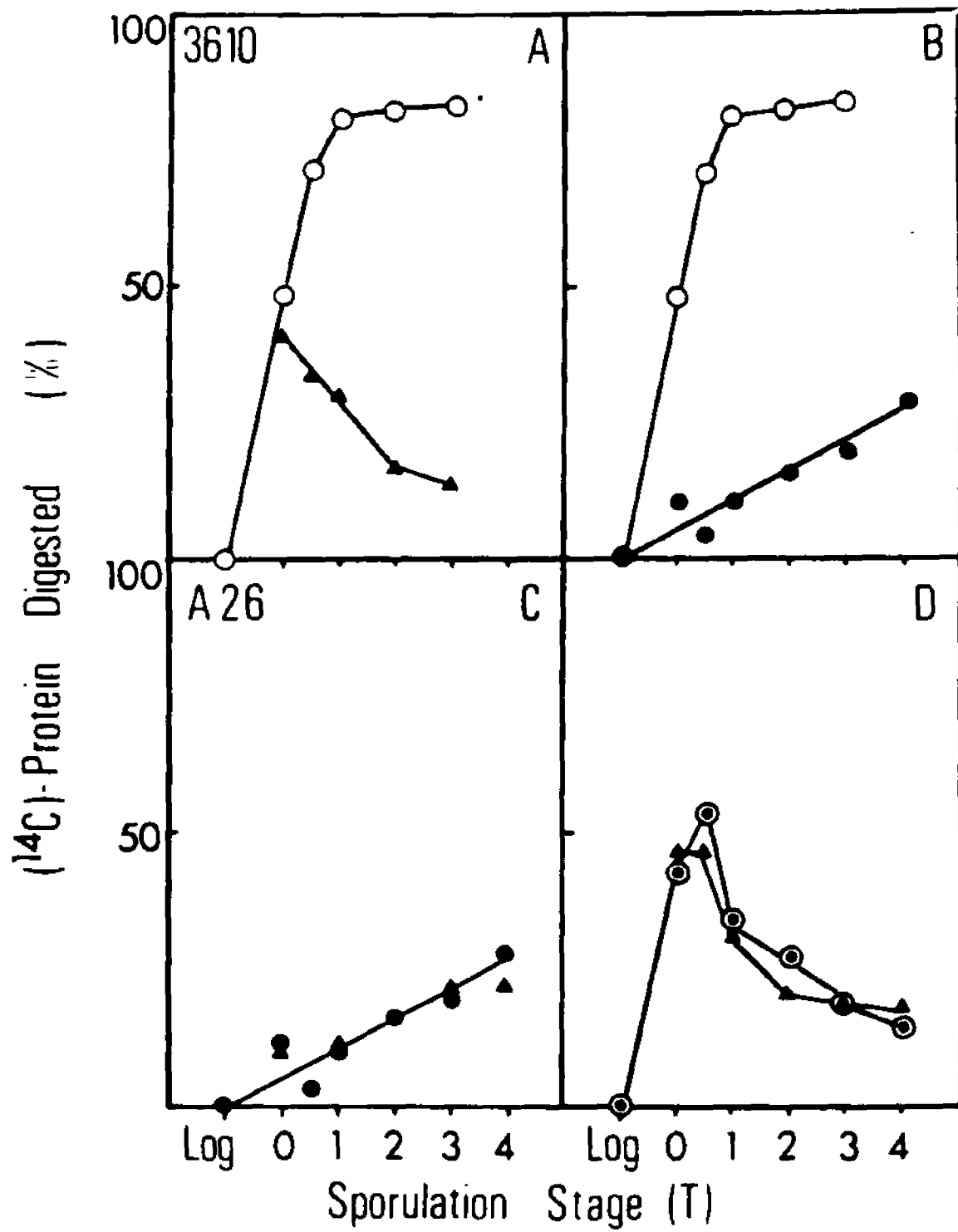


Figure 8 Relative uptake of radioisotope into RNA by Bacillus subtilis strain A26u⁻ during a carbon source shift down. Panel A: B. subtilis cells were grown in a minimal salts medium (Spizizen, 1958) supplemented with amino acids, glutamate and glucose. T₀ is designated by the arrow and represents the first observed change from the pattern of vegetative growth. At a Klett () of 100, R₀, the cells were centrifuged and resuspended in an identical medium lacking glucose (panel B) in the presence () or absence () of chloramphenicol (final concentration 200 µg/ml). At different intervals samples were removed from each culture, mixed with (³H)Uridine (5 µc /ml) for two minutes and then mixed with an equal volume of cold TMA buffer. The quantity of radioactivity determined as described in the materials and methods is expressed as that amount relative to the amount present in vegetative cells at R₀ (panel B) or in early stationary phase cells at T₀ (panel A). Symbols: acid soluble pools (+CM,  ; -CM, ), RNA (+CM,  ; -CM, ).

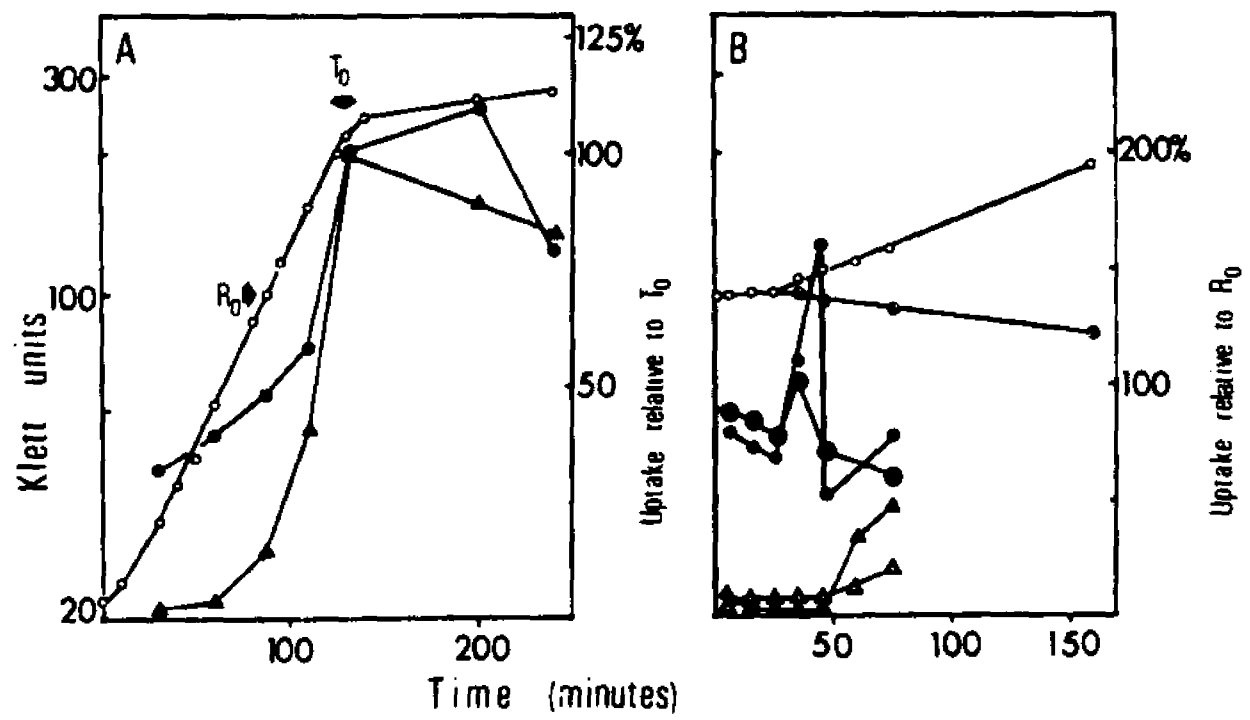


Figure 9 The rate of RNA synthesis and the soluble pool size of Bacillus subtilis A26u⁻ in R medium at stage T₂ of sporulation. A 10 ml sample was removed from a master culture of cells sporulating in R medium containing 4×10^{-4} M phosphate. The cells were mixed with (³H)Uridine and (³²P)phosphate, each at a final concentration of 35.7 μ C /ml, at T₂ (zero time). Duplicate 0.5 ml aliquots were removed at various time intervals, mixed with 10 volumes of cold TMA buffer and treated as described in Materials and Methods (procedure I). The quantity of TCA precipitable radioactivity is expressed as cpm per μ g RNA, where the RNA was determined by the orcinol reaction. Acid soluble pools are expressed as cpm per unit cell mass, ie. Klett units. The overall rate of RNA synthesis at T₂ is determined from the slope of the best fit curve as shown in panel B. The rates for (³H) and (³²P) RNA are 25 and 47 cpm/ μ g RNA per minute respectively. These represent values 11.2% and 9.9% for ³H and ³²P respectively, of the rates observed during vegetative growth. Acid soluble pools, panel A: ³²P (○) and ³H (△). TCA precipitable RNA, panel B: ³²P (●) and ³H (▲).

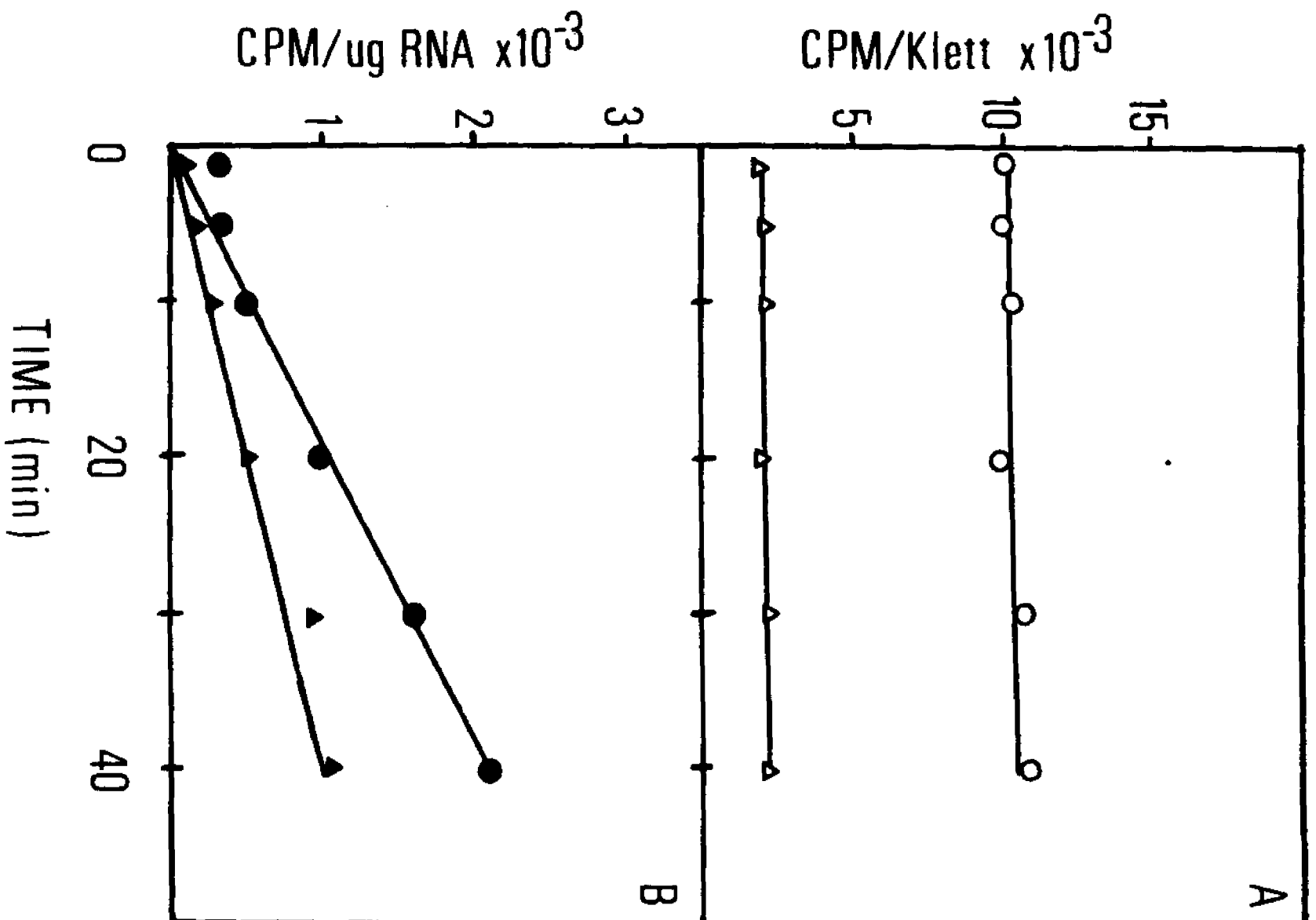


Figure 10 Analysis of the rates of RNA accumulation in Bacillus subtilis strain A26u⁻ during growth and sporulation in the Sterlini and Mandelstam sporulation media (SM-R). At different intervals 10 ml samples of the original culture were mixed with (³H)Uridine and (³²P)phosphate and followed for 40 to 45 minutes as described in Figure 9. The rates of RNA accumulation are expressed as the percent of the vegetative value (165 cpm/ug per min and 474 cpm/ug per min) for ³H (Δ) and ³²P ($\bigcirc$) respectively. The changes in the soluble pool sizes are expressed as the percent of that observed at T₀ (2,680 cpm/Klett and 11,988 cpm/klett) for ³H ($\blacktriangle$) and ³²P ($\bullet$) respectively.

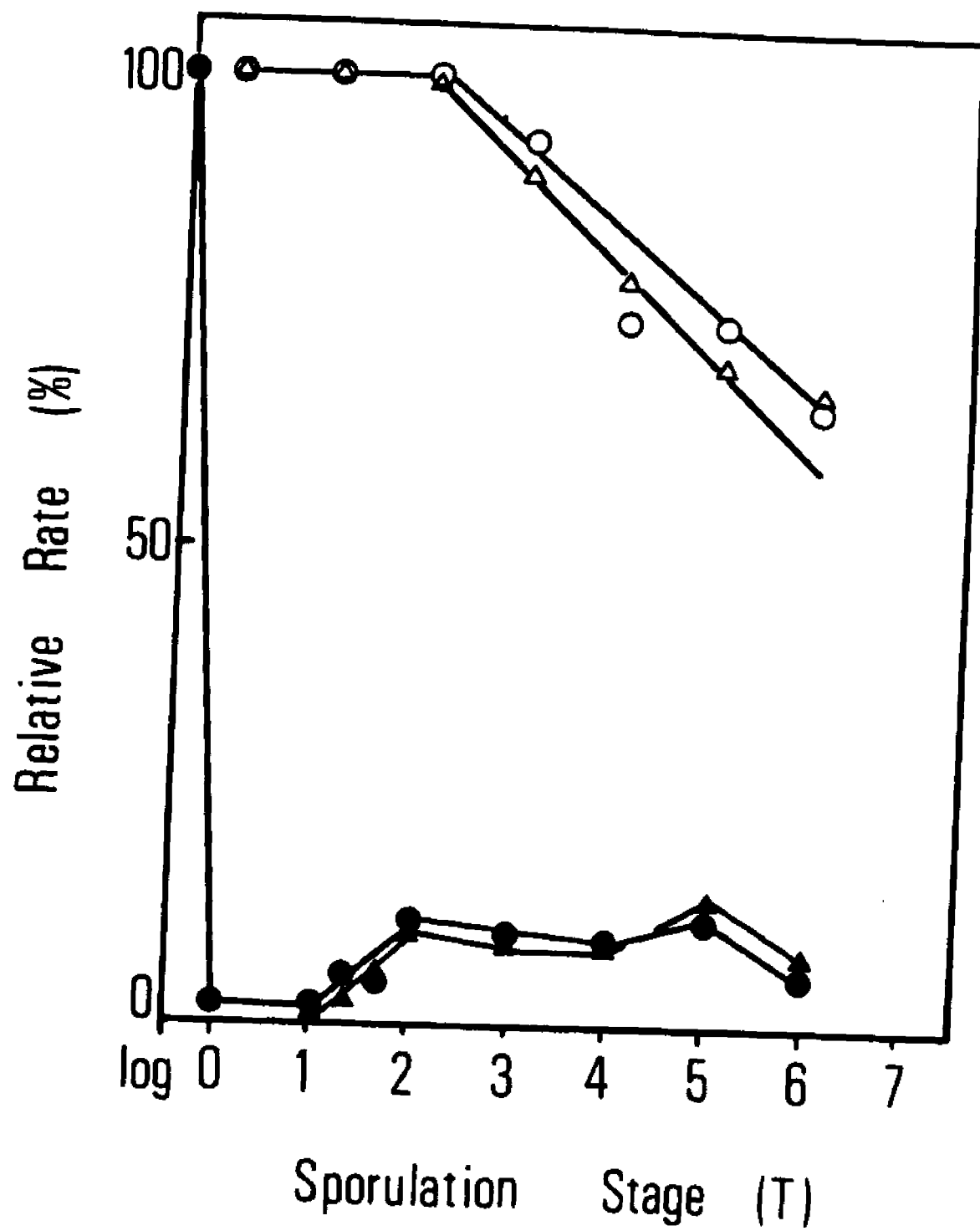


Figure 11 Relative uptake of radioisotope into RNA by Bacillus subtilis strain A26u⁻ in the double strength Shaeffer Sporulation medium (2xSG). B. subtilis cells were grown in 2xSG medium until stationary phase, centrifuged and resuspended into fresh 2xSG medium at a cell density of 10. T_0 is denoted by the vertical dashed line and is the first observable change in the logarithmic growth pattern. Changes in cell mass ($\bigcirc$) were monitored by a Klett-Summerson Colorimeter. During growth and sporulation aliquots were removed for the determination of uptake into soluble and insoluble material as described in Figure 8. The quantity of radioactivity is expressed as the percentage of that observed at $T_{0.5}$, in the acid soluble pools (Δ) and the RNA ($\blacktriangle$). The quantity of (^3H) radioactivity present at $T_{0.5}$ in the soluble pools and the RNA was 15,865 cpm and 18,325 cpm respectively.

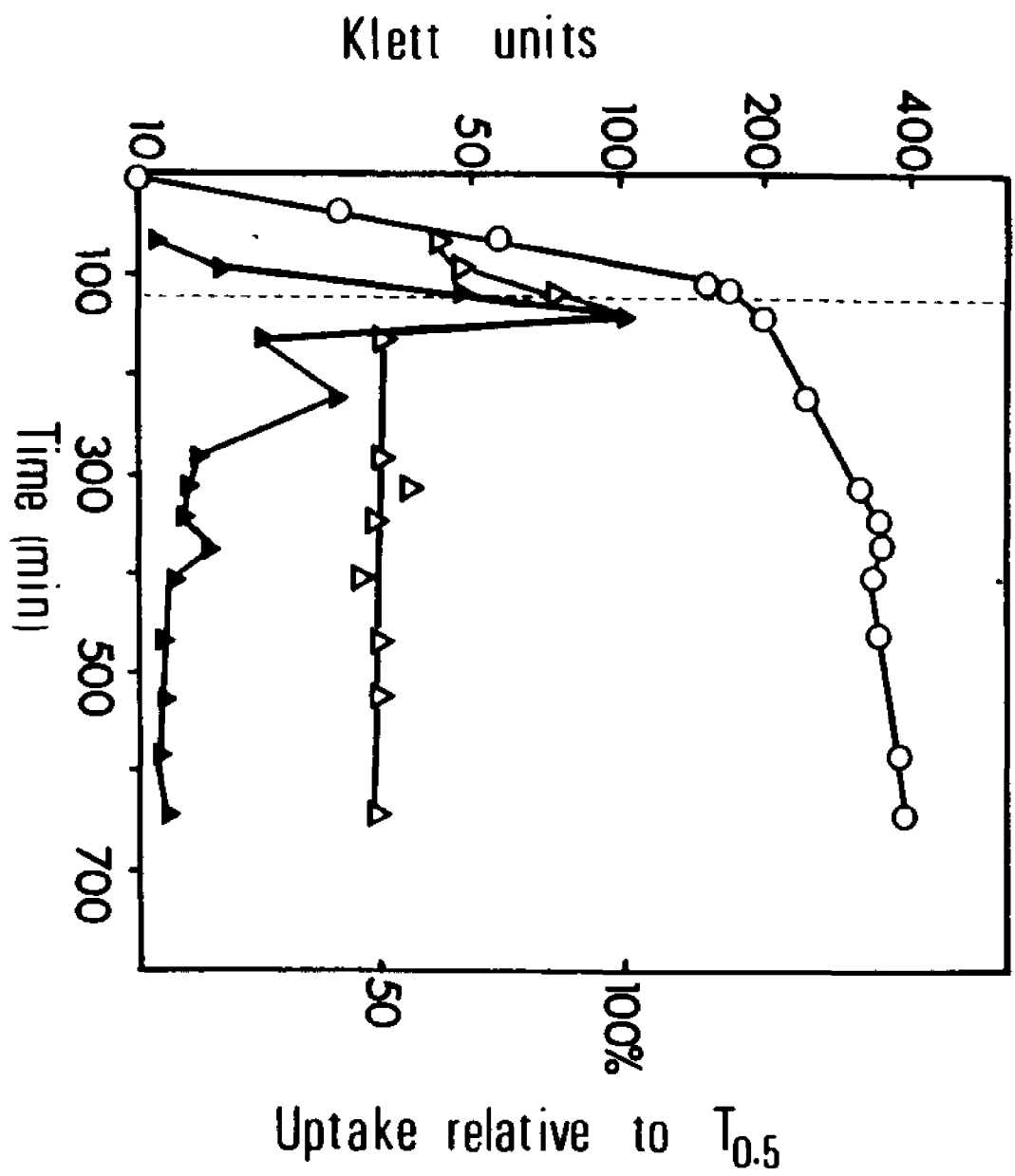


Figure 12 Relative uptake of radioisotope into RNA by Bacillus subtilis strain 3610 in the Sonenshein and Roscoe Sporulation medium (1218). Cells were grown as shown in Figure 5 in 1218 medium. Changes in cell mass ($\bigcirc$) were monitored by a Klett-Summerson Colorimeter. At different periods during growth and sporulation, aliquots were removed for the determination of uptake into soluble and insoluble material as described in Figure 8. The quantity of radioactivity is expressed as the percentage of that observed at T_0 in the acid soluble pools ($\triangle$) and the RNA ($\blacktriangle$). The quantity of (^3H) radioactivity present at T_0 in the soluble pools and the RNA was 32,985 cpm and 92,900 cpm respectively.

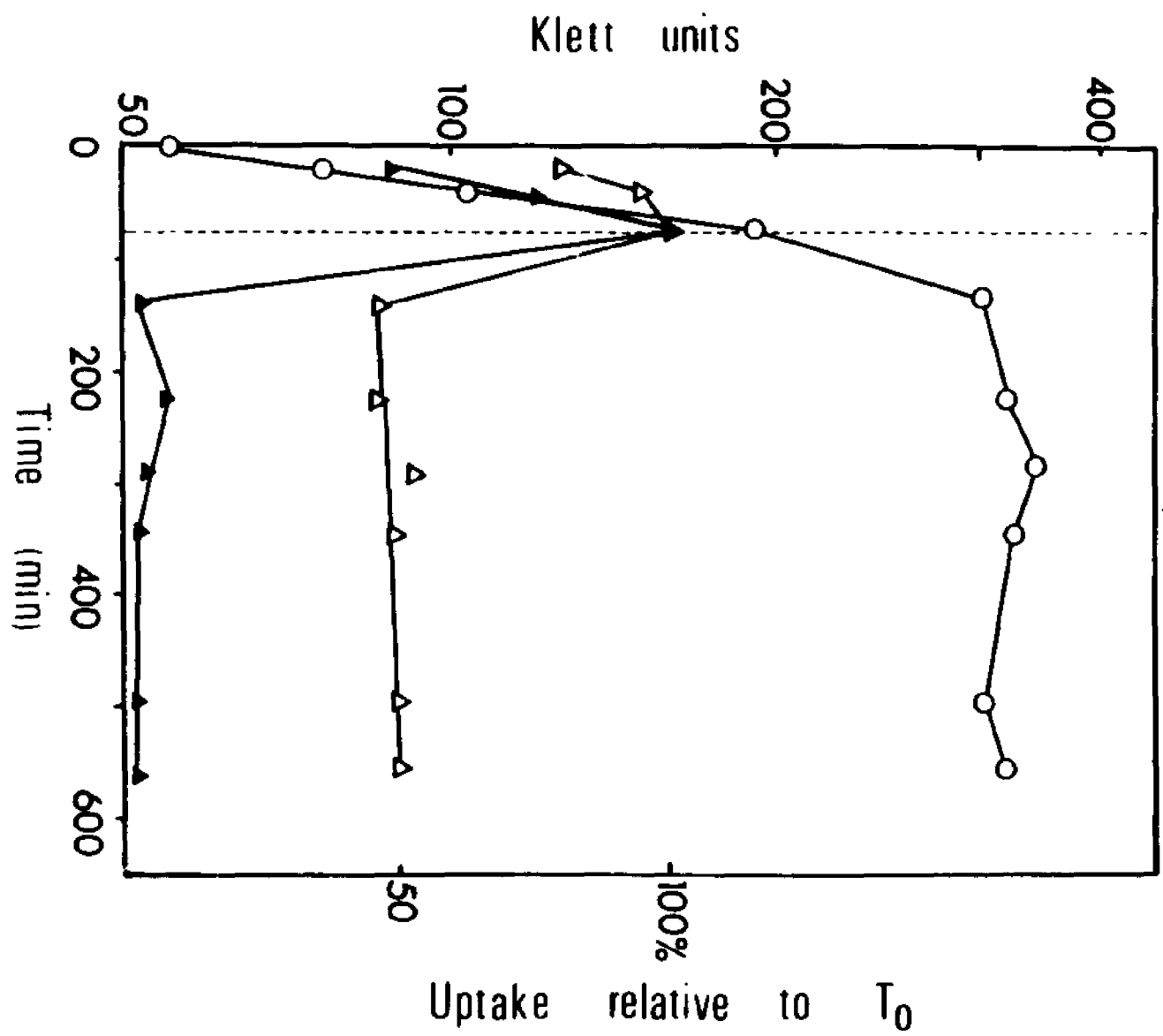


Figure 13 Relative uptake of radioisotope into RNA by Bacillus subtilis sp^+ strains in the Sonenshein and Roscoe Sporulation medium (121B). B. subtilis strains (A) A26u⁻, (B) BD29 and (C) W23 were grown in 121B medium as described in Figure 5. The mass of the culture ($\bigcirc$) was monitored by a Klett-Summerson Colorimeter. T_0 is denoted by the arrow and is the first observed change in the growth pattern. At different times during growth and stationary phase aliquots were removed for the determination of uptake into soluble and insoluble material as described in Figure 8. The quantity of radioactivity is expressed as that amount relative to that found at T_0 , in the acid soluble pools ($\triangle$) and the RNA ($\blacktriangle$). The quantity of (^3H) radioactivity present at T_0 in the soluble pools and the RNA was 121,800 cpm and 55,750 cpm for A26u⁻, 17,575 cpm and 55,230 cpm for BD29, and 66,095 cpm and 69,992 cpm for W23 respectively.

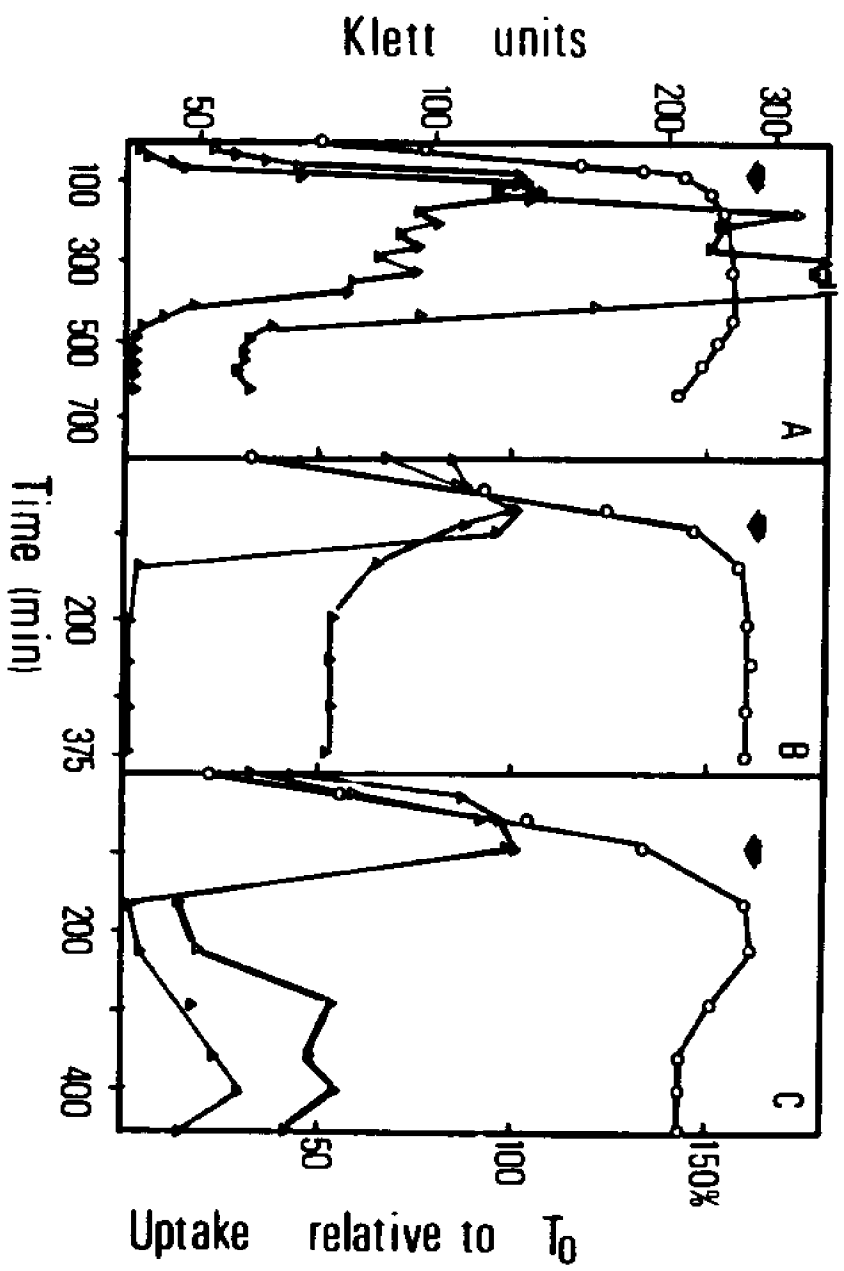


Figure 14 Relative uptake of radioisotope into RNA by Bacillus subtilis sp⁻ strains in the Sonenshein and Roscoe medium (121B). B. subtilis strains (A) SP98-8 and (B) rfm3Y were grown in 121B medium as described in Figure 5. The mass of the culture () was monitored by a Klett-Summerson Colorimeter. T₀ is denoted by the arrow and is the first observed change in the growth pattern. At different times during growth and stationary phase aliquots were removed for the determination of uptake into soluble and insoluble material as described in Figure 8. The quantity of radioactivity is expressed as that amount relative to that found at T₀, in the acid soluble pools () and the RNA (). The acid soluble pools in strain OB3Y (panel B) were observed to be much higher (160%) than the value observed at T₀. The quantity of (³H) radioactivity present at T₀ in the soluble pools and the RNA was 80,255 cpm and 69,810 cpm for SP98-8, and 36,835 cpm and 51,275 cpm for OB3Y.

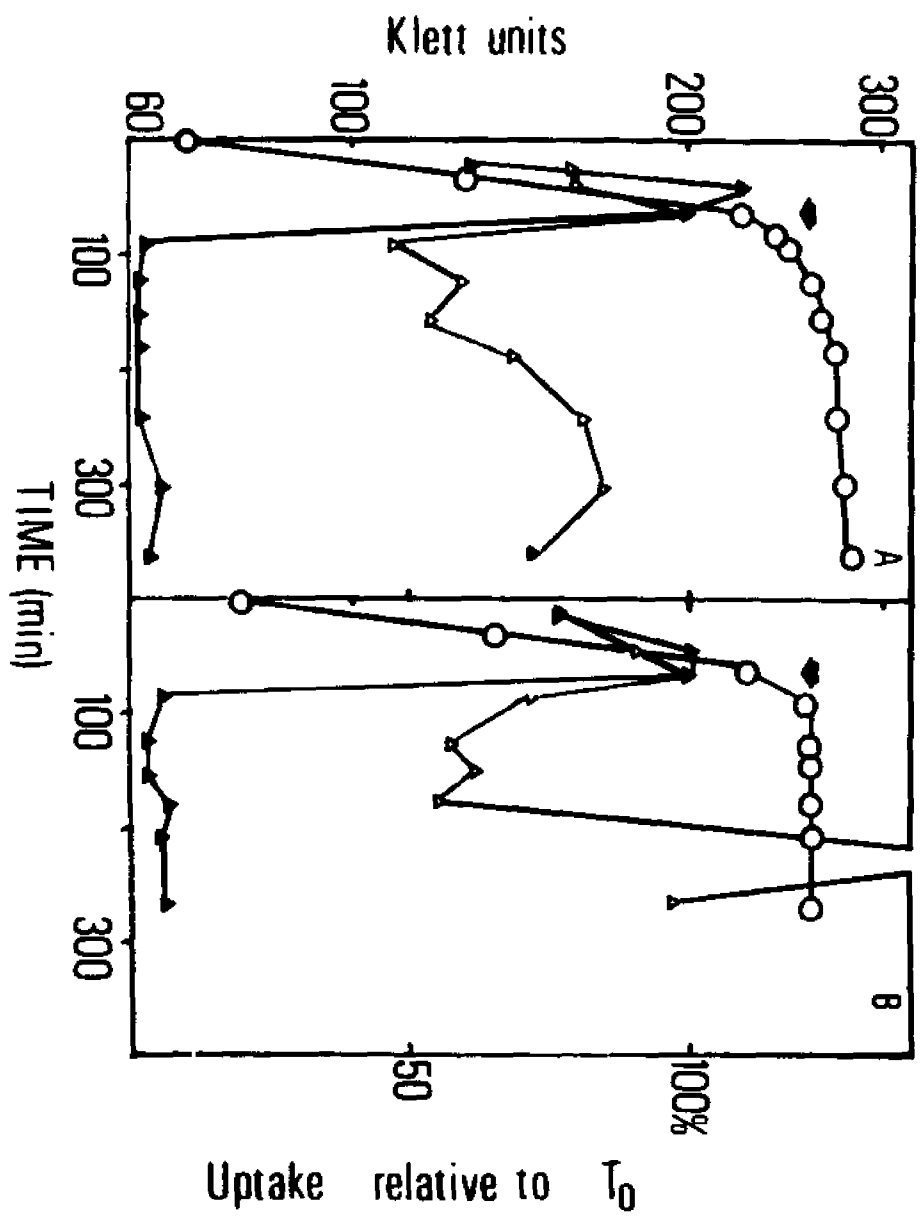


Figure 15 Chromatography of double-labeled (^3H , ^{32}P) T_2 Sporulation RNA isolated from Bacillus subtilis strains A26u⁻ grown in the Sterlini and Mandelstam media (SM-R). *B. subtilis* cells were labeled with (^3H) Uridine during growth and with (^{32}P) phosphate for 30 minutes during sporulation, as described in the materials and methods. Radioactive T_2 RNA (0.1 mg with a specific activity as cpm/ug of 5,130 and 2,160 for (^3H) and (^{32}P) respectively) was mixed with unlabeled vegetative marker RNA (1.06 mg) and applied to a MAK column (1.9 x 8.0 cm). The RNA was eluted using a linear salt gradient between 0.3 and 1.4 M sodium chloride in 0.05 M sodium phosphate buffer, pH 6.8 (total volume, 600 ml). The 4 ml fractions were collected and a 1 ml sample of each fraction was mixed with scintillation mixture (Bray, 1960). The arrows indicate the peak absorbance (A_{260}) of the unlabeled 16s and 23S marker RNAs. Recovery of the unlabeled RNA, as percent of input: total 90.7; peak I (fractions 1-24) 9.2; 4S +5S (fractions 36-60), 14.3; and 16S + 23S (fractions 72-105), 67.2 . Symbols, (^3H) label ($\bigcirc$), (^{32}P) label ($\bullet$).

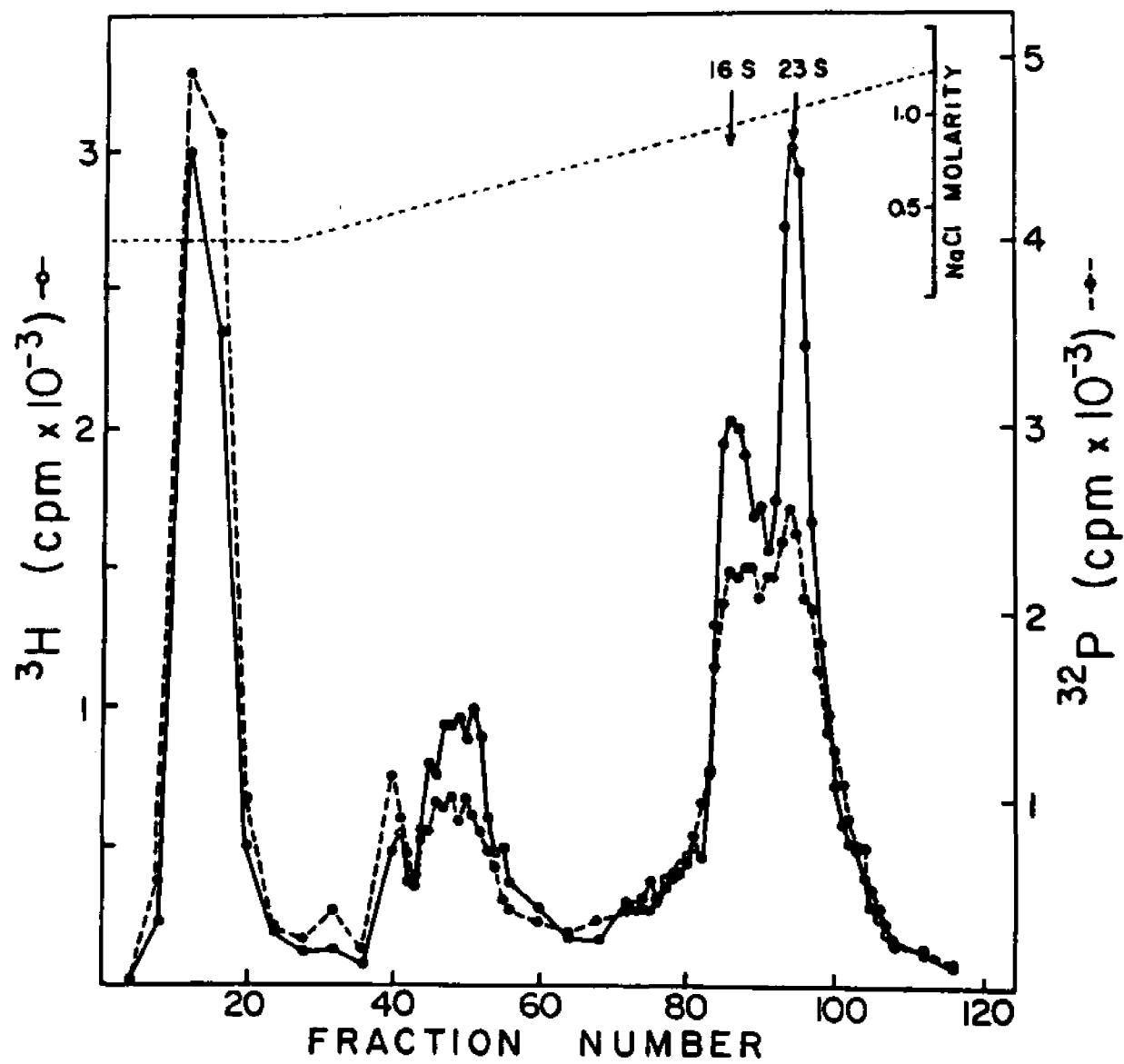


Figure 16 Kinetics of the degradation of the vegetative (^3H)labeled RNA during sporulation as assayed by the amount of (^3H) in peak I of the MAK profiles. Various doubly-labeled (^3H , ^{32}P) sporulation and (^3H) vegetative RNA preparations were chromatographed on MAK column as shown in Figure 15. The quantity of (^3H) radioactivity which appeared in the peak I region of the MAK profile was determined from the percentage of the total (^3H) RNA recovery from the column. Each determination is the average of two or more independent experiments and is presented as a function of the stage of growth or sporulation.

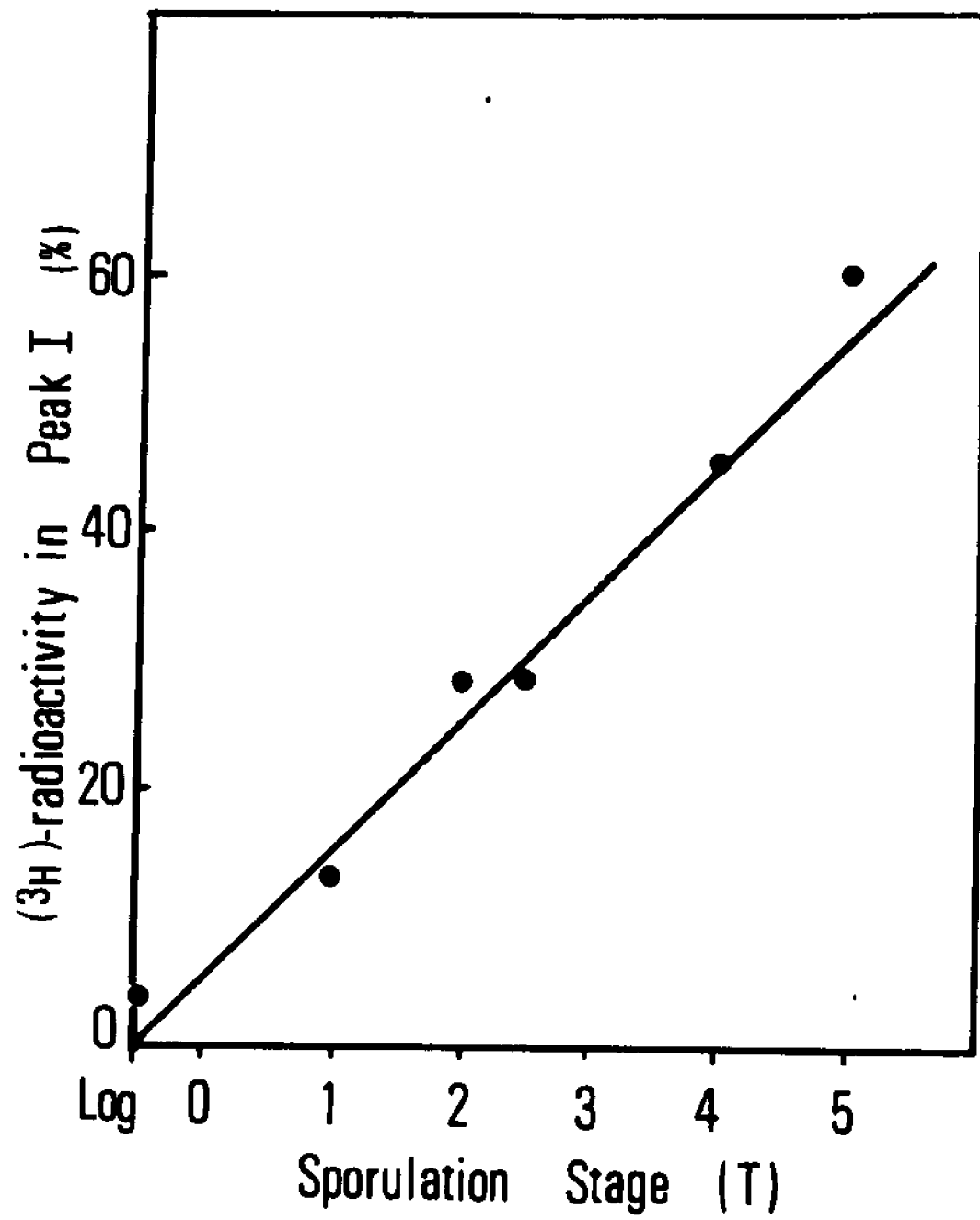


Figure 17 Chromatography of alkali denatured Bacillus subtilis DNA on a MAK column. Alkali denatured DNA (1.8 mg) isolated from B. subtilis strain A26u⁻ was applied in 0.6 M NaCl in a 0.05 M sodium phosphate buffer, pH 6.8, to a MAK column (1.9 x 8.0 cm) using 1/2 of the standard recipe, and eluted with a linear gradient of NaCl (0.6 - 1.4 M NaCl in 0.05 M sodium phosphate buffer, pH 6.8) total volume, 500 ml. The gradient was interrupted at tube 21 and reestablished at tube 26. Fractions of 5.5 ml were collected. The recovery (as percent of input) was: Total, 76.1; L, 35.0; H, 36.0. The ratio of L DNA to H DNA (L/H) was 0.97. The regions of the MAK column which were pooled for use in hybridization reaction are: L, tubes 17-28; H, tubes 31-38.

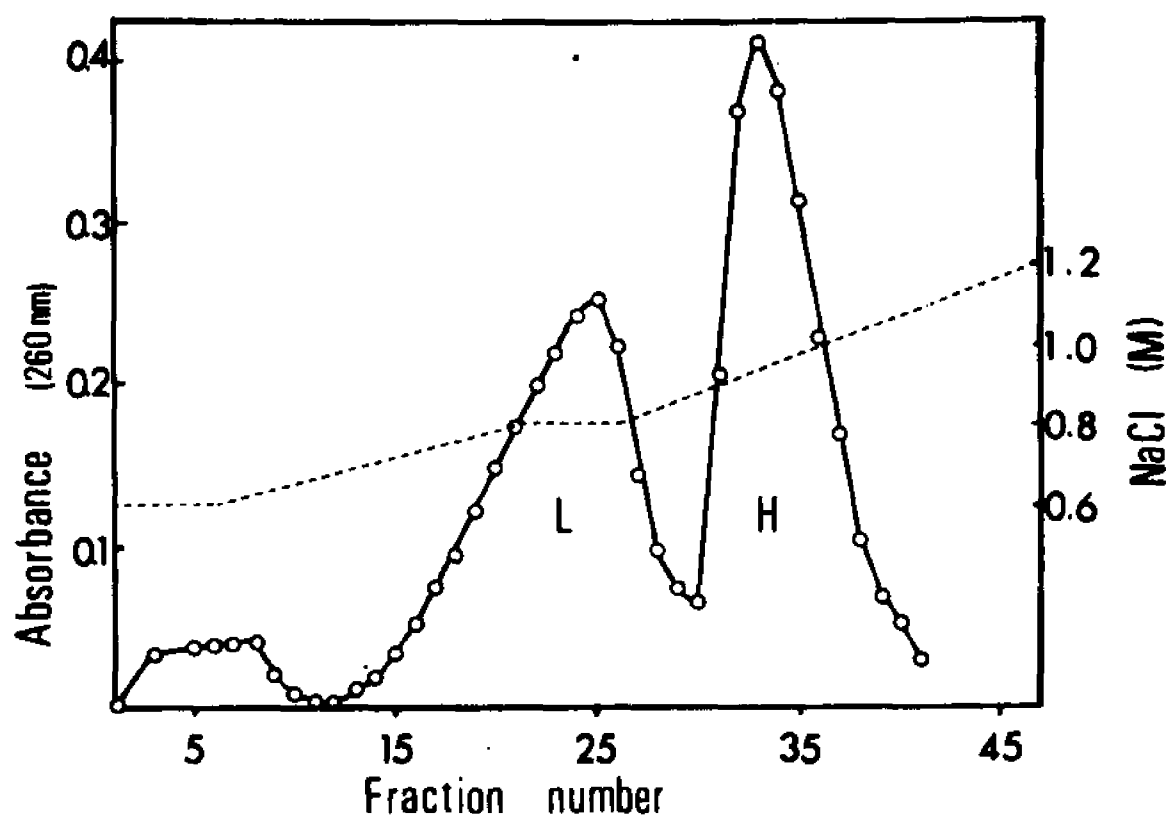


Figure 18 Fractionation of RNA isolated from purified ribosomes on a MAK column. B. subtilis strain A26u⁻ was grown in SM medium until early stationary phase, treated with rifampicin (30 ug/ml), the ribosomes isolated and the rRNA extracted as described. The rRNA (0.77 mg in 0.3 M NaCl, 0.05 M NaPO₄ buffer, pH 6.8) was applied to a MAK column prepared using 1/2 the standard recipe, and eluted using a linear salt gradient (0.3 M NaCl, 0.05 M sodium phosphate, pH 6.8; total volume, 600 ml). Fractions of 4.0 ml were collected and the absorbance of each fraction determined. The recovery (as percent of input): total, 94.9; 5S, 4.5; 16S, 31.9; 23S, 63.6. The ratio of 23S/23S was 1.99.

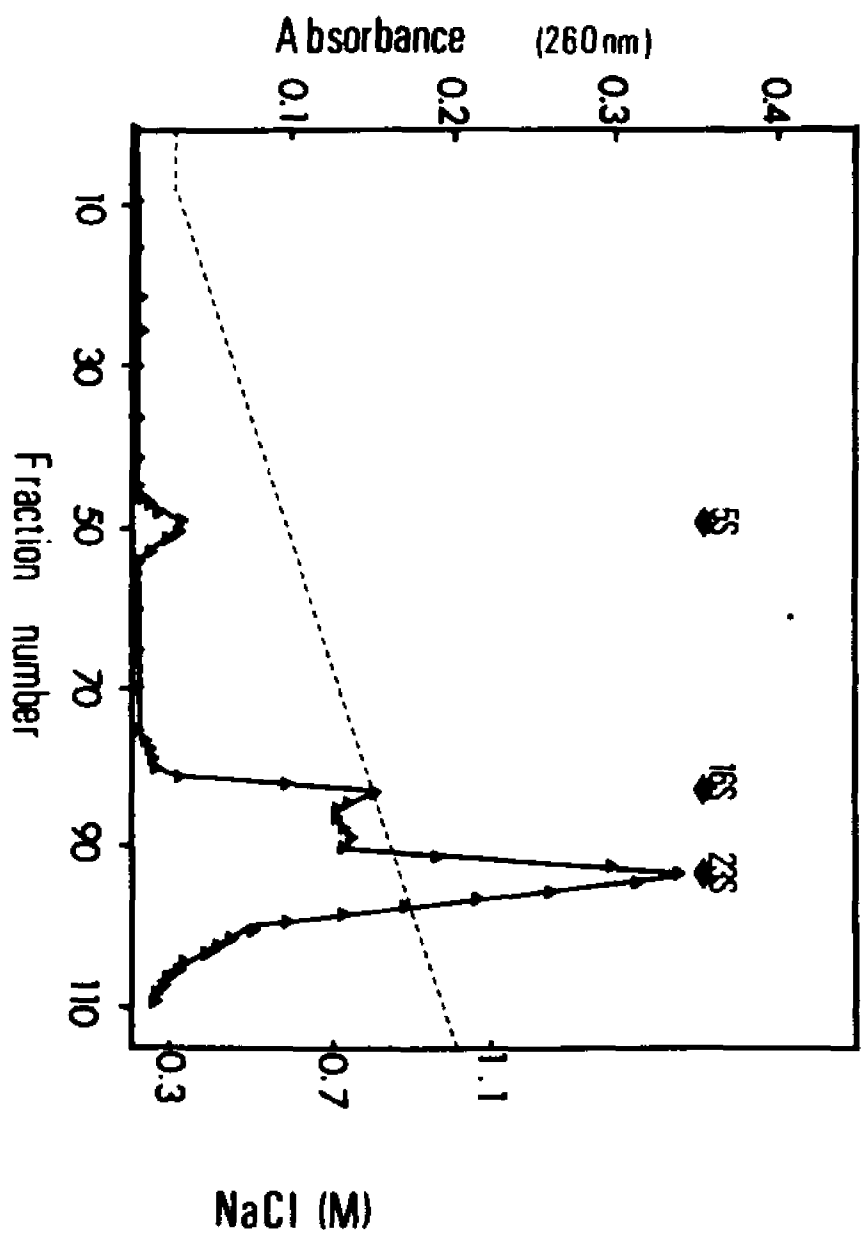


Figure 19 Fractionation of total stable RNA (srRNA) on a MAK column.

B. subtilis strain A26u⁻ was grown in Spizizen minimal salts medium.

At midlogarithmic growth Actinomycin D (10 μ g/ml) was added, the cells harvested and the RNA extracted as described in the Materials and Methods. The srRNA (1.44 mg in 0.3 M NaCl, 0.05 M sodium phosphate buffer, pH 6.8) was applied to a MAK column using the full recipe, eluted with a linear salt gradient (0.3-1.4 M NaCl, 0.05 M sodium phosphate buffer, pH 6.8; total volume, 600 ml) and collected in 4 ml fractions. The gradient was interrupted at tube 76 and re-established at tube 83. The recovery(as percent of input): total, 80.7.

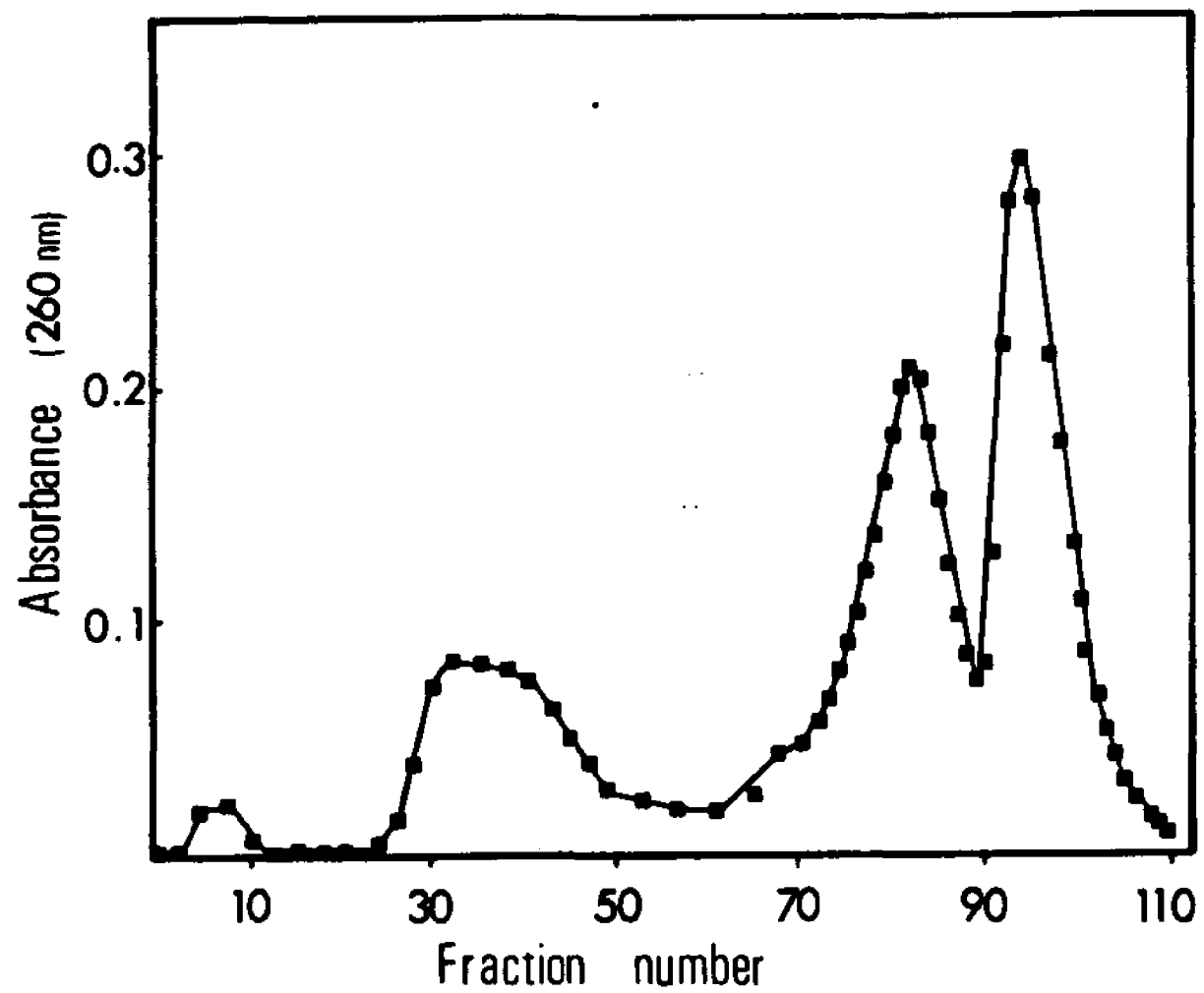


Figure 20 Hybridization of MAK-fractionated L and H DNA strands of Bacillus subtilis strain A26u⁻ to RNA labeled with (³H)uridine during vegetative growth and (³²P)phosphate during sporulation. Each annealing mixture contained labeled RNA and increasing amounts of L or H DNA in a final salt concentration of 6xSSC. The reaction mixtures in a volume of 3 ml, were incubated at 67° for 18 hours. (A) 0.07 µg of three minute pulse-labeled RNA isolated from cells grown in minimal medium (Spizizen, 1958) (log (3) RNA, specific activity as cpm/µg, 55,200) is incubated with L or H DNA. (B) 0.35 µg of eight minute pulse-labeled RNA isolated from cells grown in SM medium (log (8) RNA, specific activity as cpm/ug, 4,630) is incubated with L and H DNA. (C) 0.5 ug of double-labeled T₂ sporulation RNA isolated from cells grown in SM medium in the presence of (³H)Uridine (10 µc /ml) for four generations and labeled for 30 minutes with ³²P-phosphate (60-70 uci/ml) during sporulation in R medium (specific activity as cpm/ug, 5,130 and 2,162 for ³H and ³²P respectively) incubated with L and H DNA. (D) 0.5 ug of double-labeled T₄ sporulation RNA labeled as described in (C) above, (specific activity as cpm/µg, 4,400 and 5,110 for ³H and ³²P respectively) incubated with L and H DNA. Symbols: H-DNA (○, ●), L-DNA (□); dashed line (---), ³²P-RNA; solid line (—), ³H-RNA. The quantities of input RNA hybridized to the L-DNA strand, at a DNA/RNA ratio of 50:1, were 2.0, 3.0% 2.7% and 2.5% for panels A, B, C and D respectively.

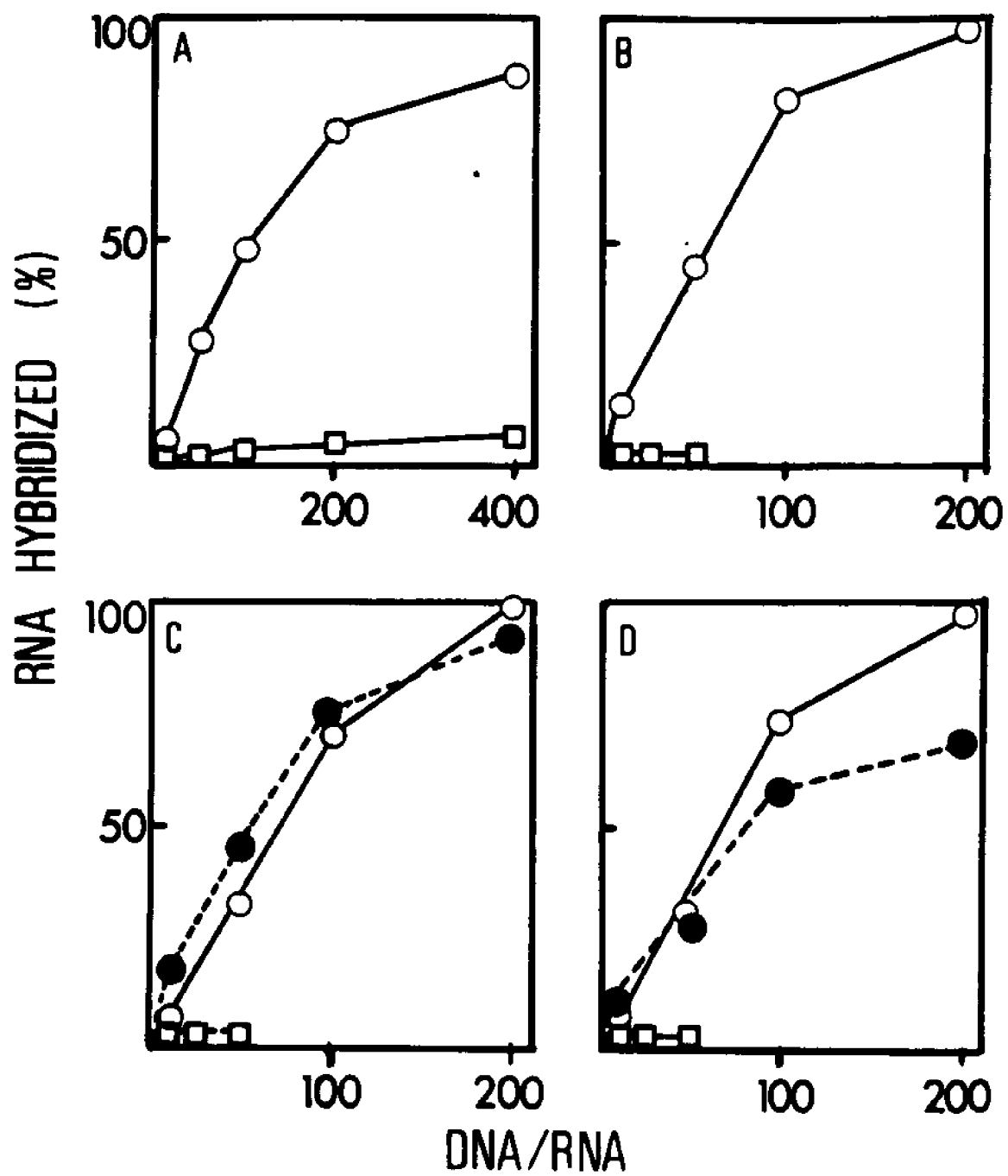


Figure 21 Hybridization of MAK-fractionated L and H DNA strands of Bacillus subtilis asporogenous strains SP98-8 and OA5NA to RNA labeled with ^3H Uridine during vegetative growth and ^{32}P phosphate during stationary phase. Each annealing mixture contained labeled RNA and increasing amounts of L or H DNA strands in a final salt concentration of 6xSSC and was incubated at 67° for 18 hours. The hybrids were collected and treated as described in the Materials and Methods.

(A) SP98-8 double-labeled $T_{2.5}$ RNA (specific activity as cpm/ μg , 347, 234 and 7,723 for ^3H and ^{32}P respectively) was isolated from cells grown in SM medium in the presence of ^3H Uridine ($25 \mu\text{C} / \text{ml}$) for four generations and labeled for 30 minutes at $T_{2.5}$ during stationary phase in R medium with ^{32}P phosphate ($50 \mu\text{C} / \text{ml}$). The amounts of RNA incubated with increasing amounts of purified H and L DNA were $0.35 \mu\text{g}$ and $0.70 \mu\text{g}$ in final volumes of 1.26 ml and 2.28 ml respectively.

(B) OA5NA double-labeled RNA (specific activity as cpm/ μg , 492,728 and 60,994 for ^3H and ^{32}P respectively) was labeled at $T_{2.5}$ as described above in (A). The amounts of RNA incubated with increasing amounts of purified H and L DNA were 0.16 and 0.64 μg in final volumes of 1.26 ml and 2.28 ml respectively. Symbols: H-DNA ($\bigcirc$, $\bullet$), L-DNA ($\square$); dashed lines, ^{32}P RNA; solid lines, ^3H RNA.

RNA HYBRIDIZED (%)

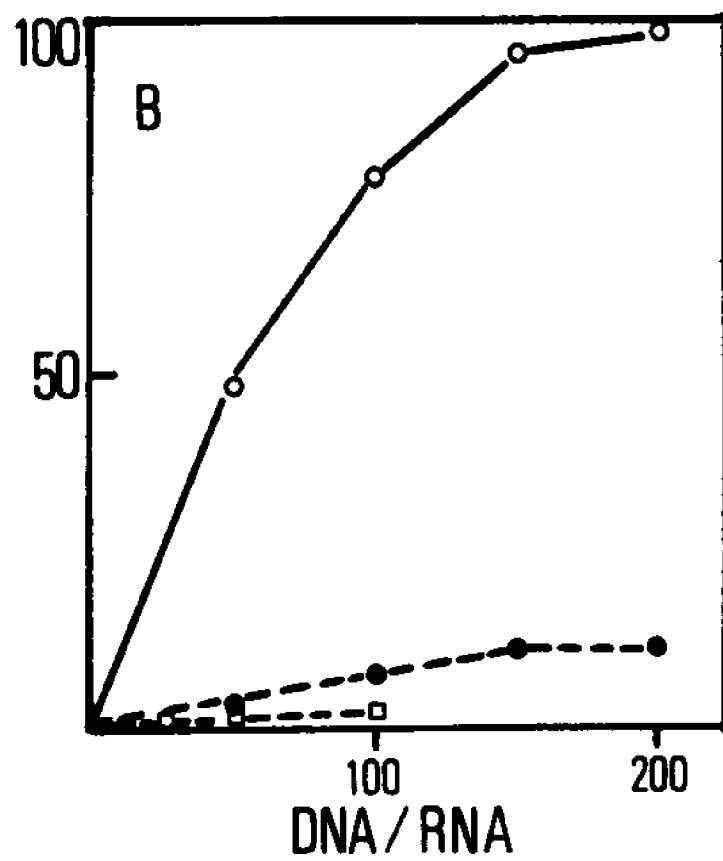
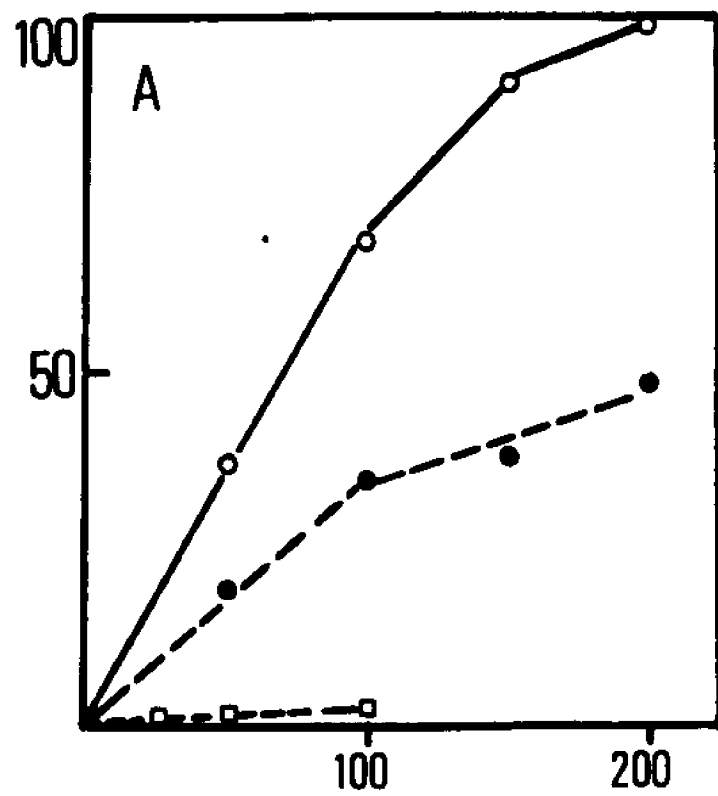


Figure 22 Hybridization of separated DNA strands of Bacillus subtilis strain A26u⁻ with(³H)labeled RNA isolated from vegetative and sporulating cells at stage T₂. B. subtilis cells were grown in SM-R media and labeled with(³H)Uridine (200 µc /ml) during vegetative growth and sporulating (T₂) for three minutes. The RNA was isolated from 1 ml of cell culture according to the Summers procedure as described (Summers, 1969). Alkali denatured DNA from strain A26u⁻ (5.75 mg in 0.65 M NaCl, 0.05 M sodium phosphate buffer, pH 6.8) was applied to a standard size MAK column and eluted with a linear salt gradient (0.65-1.5 M NaCl, 0.05 M sodium phosphate buffer, pH 6.8; total volume 500 ml). The gradient was interrupted (D) at tube 48 and reestablished (R) at tube 55. Recovery(as percent of input): total, 86.6; L, 43.4; H, 33.8; the L/H ratio was 1.28. The pooled fractions are designated by the same letter on the elution profile. Each hybridization mixture (1.42 ml) contained 7.5 µg DNA (dialyzed against 0.1xSSC), 0.1 µg(³H) labeled RNA (Specific activity, as cpm/ug: vegetative RNA, 59, 050; T₂ sporulation RNA, 7,740) in a 6xSSC environment. Symbols: Absorbance at 260 nm, (○), RNA Hybridized (%): Vegetative, solid bars; T₂ sporulation, broken bars.

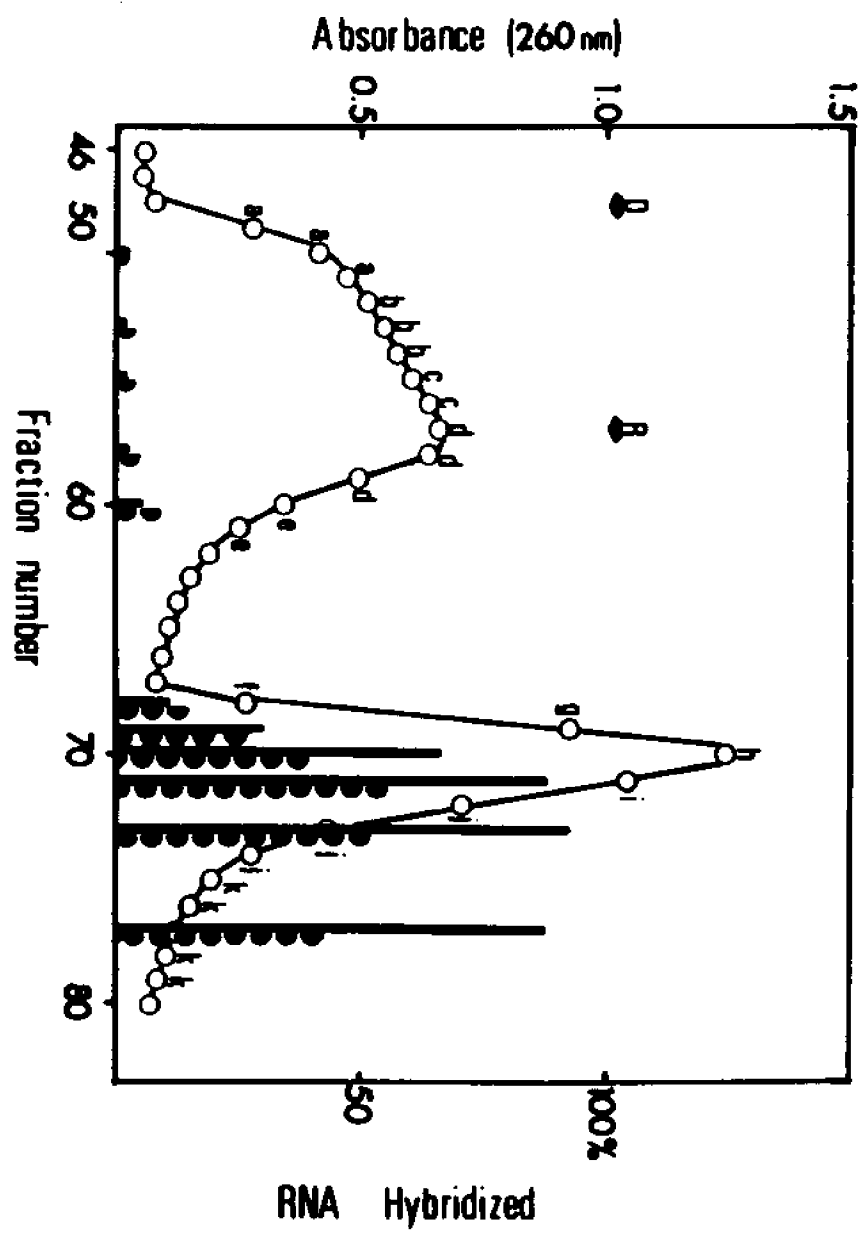


Figure 23 Correlation between the quantity of ribosomal RNA synthesized during vegetative growth and the doubling time of the cell population. Bacillus subtilis strain A26u⁻ was labeled for three minutes with(³H)Uridine during vegetative growth in Spizizen minimal salts medium, 121B, 2xSG and SM media. The RNA was extracted and the quantities of ribosomal RNA determined from competition hybridization experiments as described in the Materials and Methods. The quantity of rRNA is presented here as a function of the doubling time (μ) of the culture. Statistical determinations were made for the determination of best-fit line (linear regression analysis) and the correlation of these two variables.

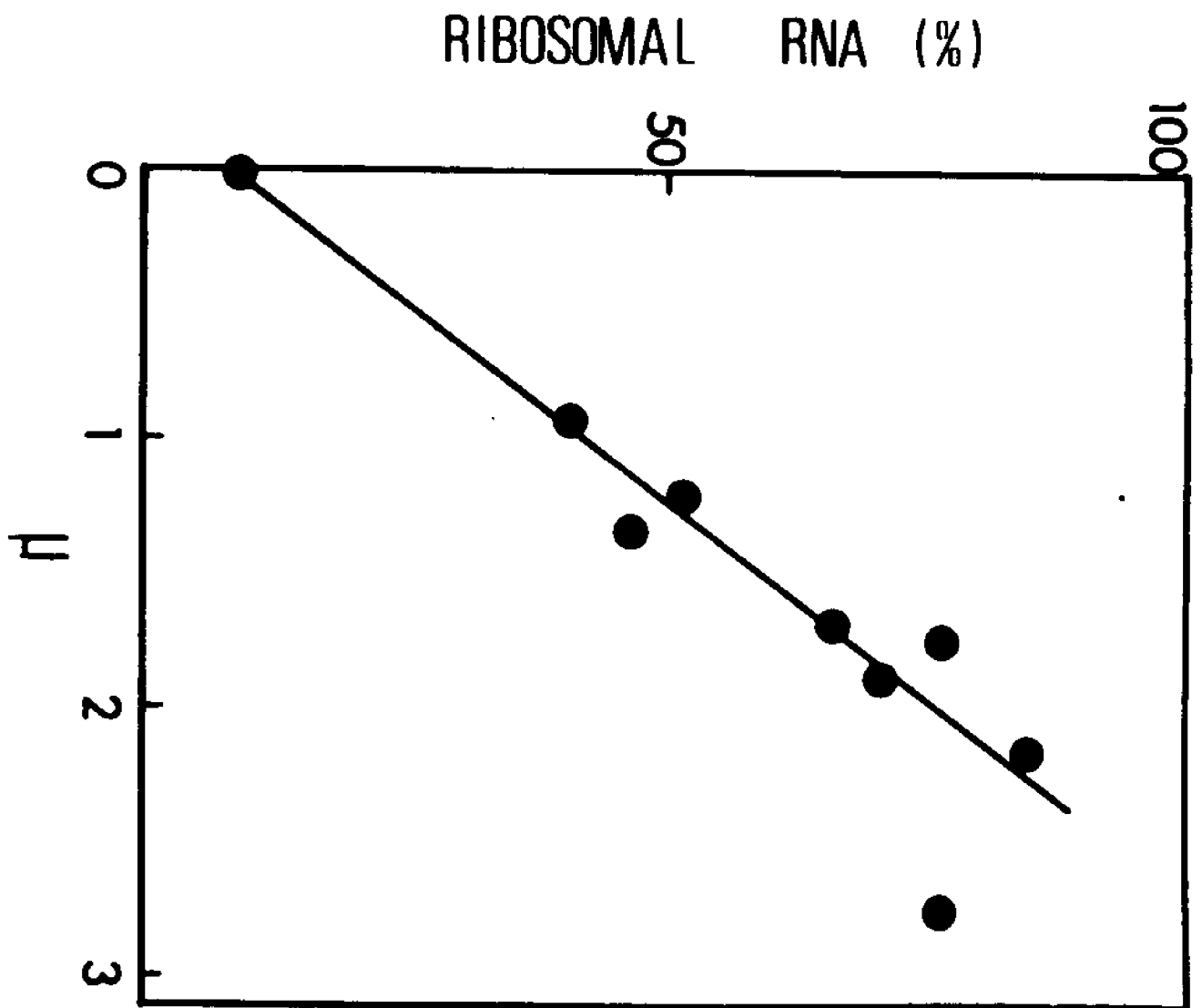


Figure 24 The influence of Chloramphenicol on the rate of RNA accumulation by Bacillus subtilis during vegetative growth and sporulation. (A) B. subtilis strain A26u⁻ during vegetative growth in medium 121B. At mid-logarithmic growth, the culture was divided in two equal portions. Each received (³H)Uridine (10 μ c /ml) and only one received chloramphenicol (200 μ g/ml). Samples were removed at various times afterward, precipitated with cold TCA (final concentration 5%), collected and the radioactivity determined. (B) B. subtilis strain 3610, was grown in medium 121B until 20 minutes prior to T₂ of stationary phase. At that time, the culture was divided in two portions, each received (³H)Uridine (10 μ c /ml) and one received chloramphenicol (200 μ g/ml). Samples removed precipitated as described in (A) above. Stage T₂ is represented by 20 minutes in panel B. Symbols: (●) in the presence of CM; (⊙) in the absence of CM.

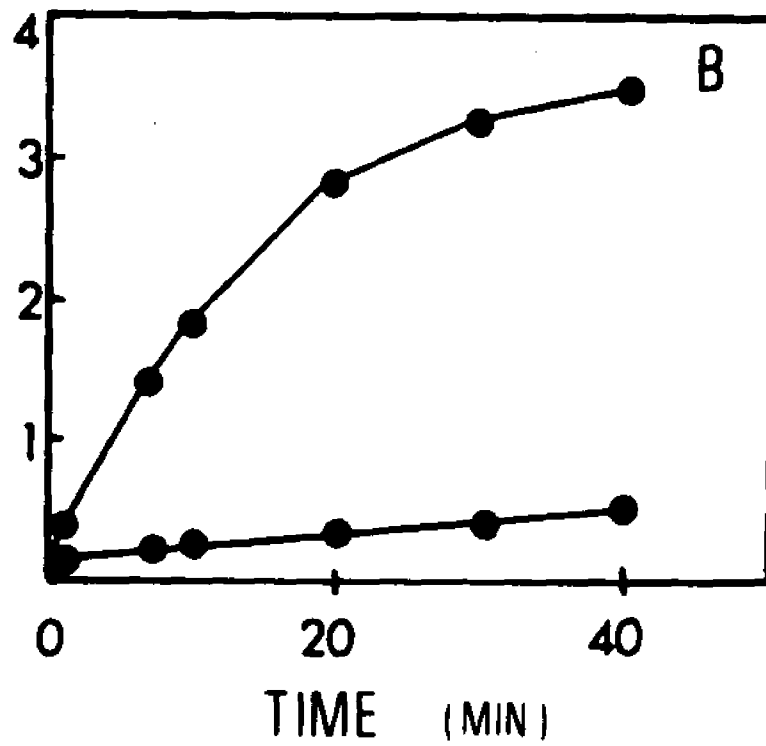
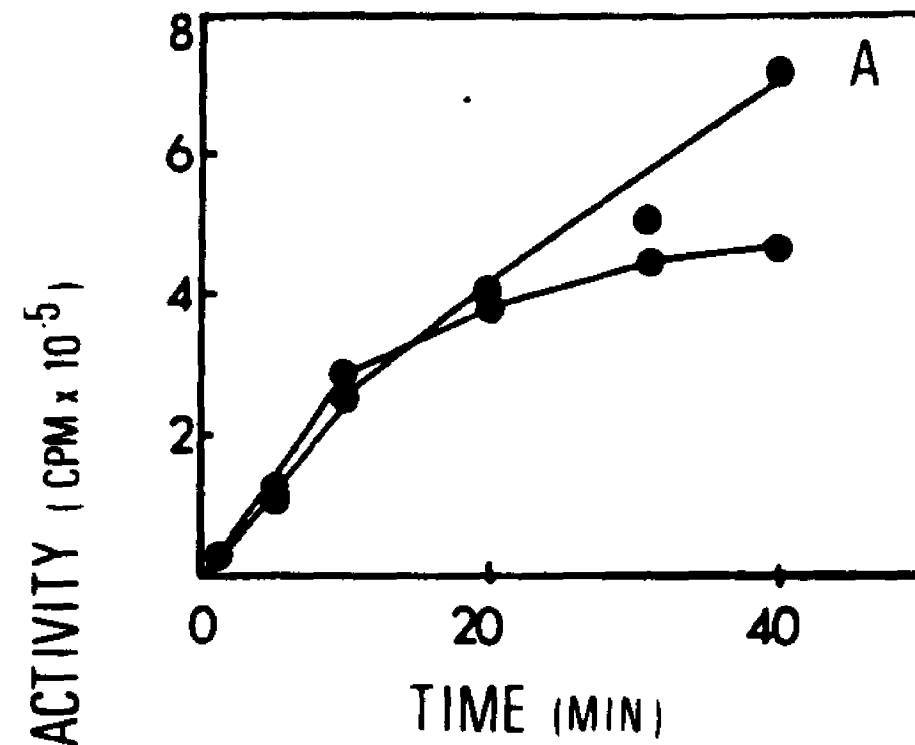


Figure 25 Hybridization of MAK-fractionated L and H DNA strands of Bacillus subtilis strain A26u⁻ with (³H)RNA isolated from cells during vegetative growth and stationary phase in the Sonenshein and Roscoe Sporulation medium. Strain A26u⁻ was grown and labeled with (³H)Uridine (50 µc /ml) for three minutes in vegetative growth, and at T₂ of stationary phase, in the presence or absence of chloramphenicol (200 µg/ml, added 20 minutes prior to labeling). Annealing mixtures contained 0.6 µg of L or H DNA strands and increasing amounts of (³H)labeled RNA in a final salt concentration of 6xSCC in a total volume of 0.3 ml. (A) (³H)labeled vegetative RNA (specific activity as cpm/ug, 15,780). (B) (³H)labeled T₂ stationary phase RNA (specific activity as cpm/µg, 30,630). (C) (³H)labeled T₂-CM stationary phase RNA made in the presence of pre-treatment with chloramphenicol (200 µg/ml) (specific activity as cpm/µg, 31,040). Symbols: H strand DNA (●), L strand DNA (▲).

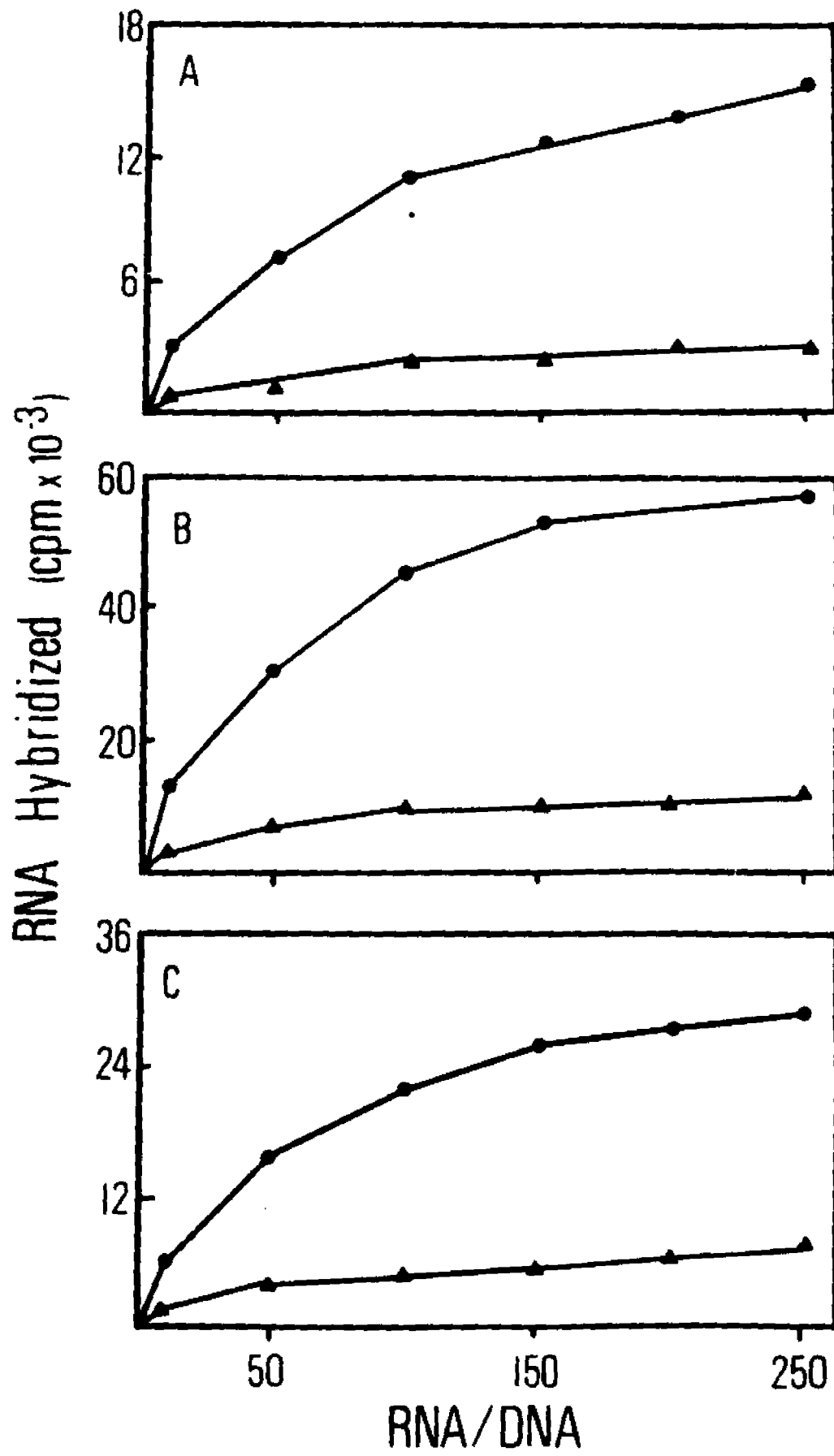


Figure 26 Competition of unlabeled RNA with (^3H) labeled RNA from the same stage at high DNA/RNA hybridization ratios. RNA was isolated from Bacillus subtilis strain A26u⁻ during vegetative growth and sporulation in the Sterlini and Mandelstam Sporulation media. These are the same RNA preparations as presented in Figure 18. Each annealing mixture contains labeled RNA, L or H DNA strands, in a final salt concentration of 6xSSC, and increasing quantities of homologous unlabeled RNA in a total volume of 0.68 ml. Incubated at (A) (^3H) Log (3) RNA isolated from vegetative cells (0.06 μg ; specific activity as cpm/ μg 55,200) incubated with 12 μg of DNA L or H strands. (B) (^3H -) Log (8) RNA isolated from vegetative cells (specific activity as cpm/ μg , 4,630): 0.35 μg RNA incubated with H-DNA (70.0 μg); 1.5 μg RNA incubated with L-DNA (75 μg). (C) (^3H , ^{32}P) labeled T₂ sporulation RNA (specific activity as cpm/ μg , 5,130 and 2,162 for (^3H) and (^{32}P) respectively): 0.5 μg RNA incubated with H-DNA (100 μg); 1.5 μg RNA incubated with L-DNA (75 μg). (D) (^3H , ^{32}P) labeled T₄ sporulation RNA (specific activity as cpm/ μg , 4,400 and 5,110 for (^3H) and (^{32}P) respectively): the same quantities of DNA and RNA were used here as in panel C above. The 100% hybrid, as cpm remaining, with no competition unlabeled RNA for H and L was respectively: (A) 2,497 and 200 (B) 1,610 and 235 (C) (^3H) 2,570, (^{32}P) 1,025 and (^{32}P) 1,777 and (^{32}P) 263. Symbols: H strand DNA (○, ●), L strand DNA (□, ■); dashed line, (^{32}P) RNA; solid line (^3H) RNA.

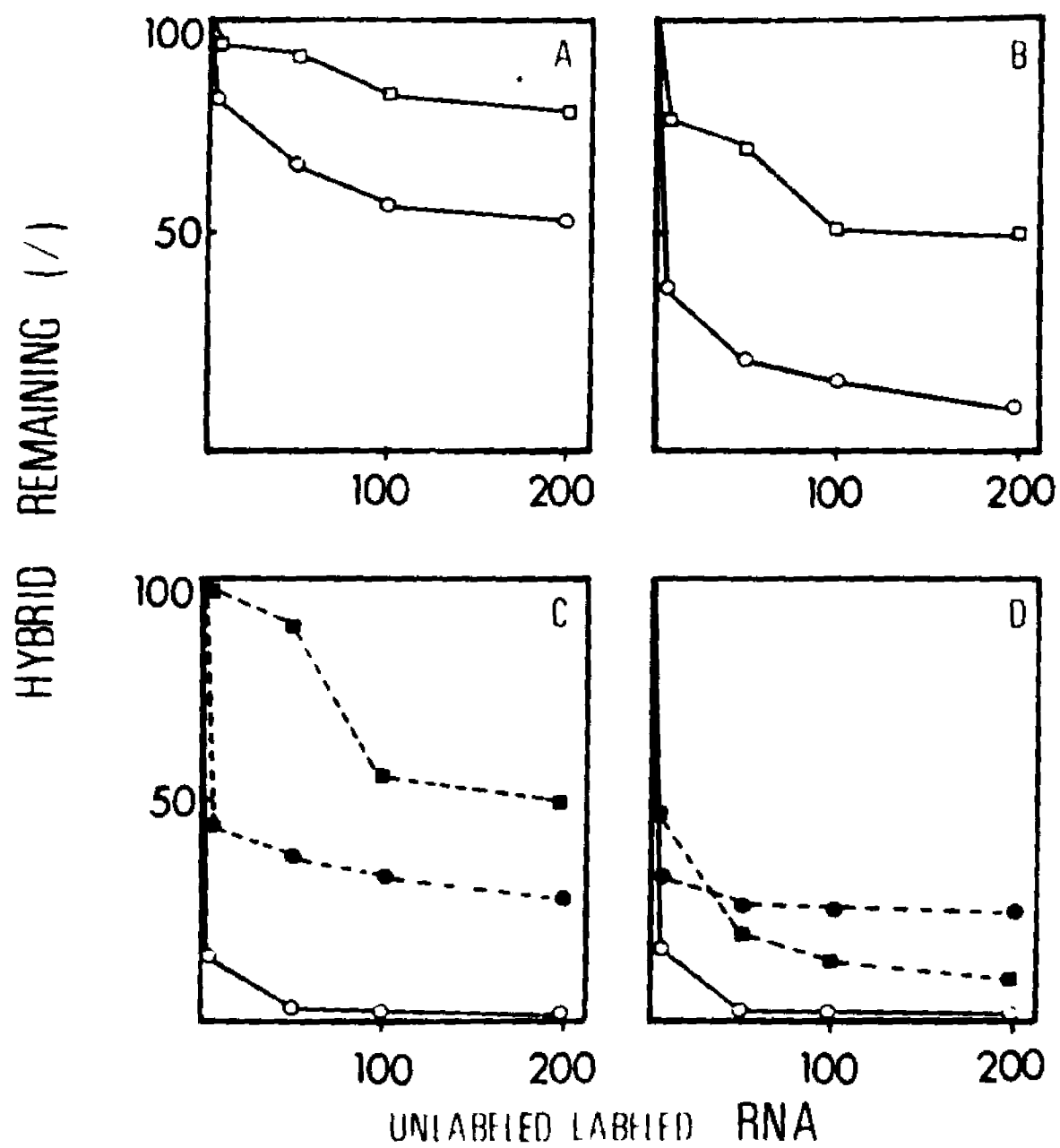


Figure 27 Competition of unlabeled RNA with (^3H) labeled RNA from the same stage at high DNA/RNA hybridization ratios. Pulse-labeled RNA was isolated from Bacillus subtilis strain A26u⁻ during vegetative growth and stationary phase in the Sonenshein and Roscoe Sporulation medium. The RNA preparations described in Figure 25 were used for this experiment. Each annealing mixture contained L (30 μg) or H (15 μg) DNA. (^3H)labeled RNA (0.3 μg or 0.1 μg for L and H assays respectively) and increasing amounts of homologous unlabeled bulk RNA in a final salt concentration of 6xSSC, in total volumes of 0.24 and 0.27 ml for H and L DNA strand reactions respectively. The reaction mixtures were incubated at 67⁰ for 18 hours. (A) (^3H)labeled vegetative RNA (specific activity as cpm/ μg , 15,780). (B) (^3H) labeled T₂-stationary phase RNA (specific activity as cpm/ μg , 30,630). (C) (^3H)labeled T₂-CM stationary phase RNA (specific activity as cpm/ μg , 31,040). The 100% hybrid, as cpm remaining with no competitor unlabeled RNA for H and L was respectively: (A) 1,465 and 145, (B) 2,748 and 430, (C) 2,511 and 475. Symbols: H strand DNA competitions (●), L strand DNA competitions (■).

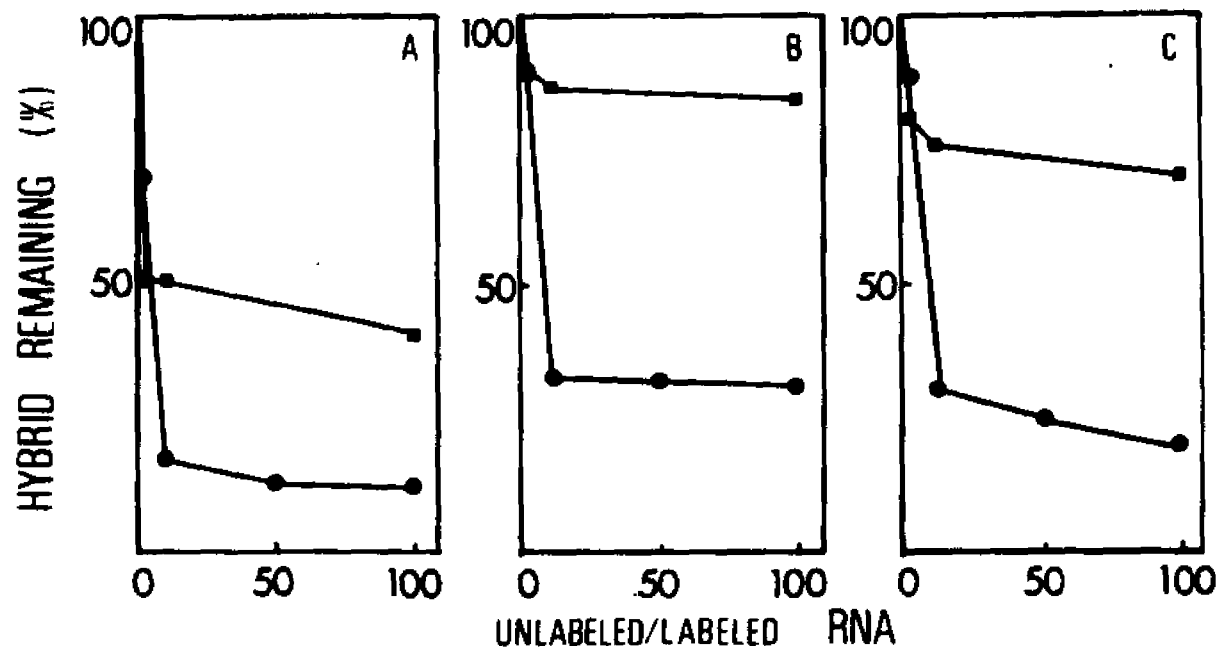


Figure 28 Competition between vegetative and sporulation RNAs from stages T_2 and T_4 during hybridization with H and L DNA strands at high RNA/DNA ratios. Labeled RNA isolated from Bacillus subtilis strain A26u⁻ during growth and sporulation in the Sterlini and Mandelstam Sporulation media was annealed to individual DNA strands at a RNA/DNA ratio of 10:1 in the presence of increasing amounts of unlabeled competitor RNA. Each annealing mixture contained 10 μ g of labeled RNA and 1 μ g of H-DNA, or 20 μ g of labeled RNA and 2 μ g of L-DNA, a final salt concentration of 6xSSC and increasing amounts of unlabeled vegetative, T_2 or T_4 competitor RNA. Panels

(³H)labeled Log (B) RNA (specific activity as cpm/ μ g, 4,630): (A) H-DNA, (B) L-DNA. Sporulation T_4 RNA described in the legend to Figure 20 (specific activity as cpm/ μ g, 4,400 and 2,836 for (³H) and (³²P) respectively): (C) H-DNA, (D) L-DNA. The 100% hybrid, as cpm, remaining, with no unlabeled competitor RNA was respectively: (A) H-DNA, 1703, (B) L-DNA, 526, (C) H-DNA, 795, (D) L-DNA, 515. The 100% values for T_2 sporulation RNA (not shown) for H and L DNA were 850 and 203 cpm respectively. Symbols for unlabeled competitor RNA preparations: vegetative (●); T_2 -sporulation (▲); T_4 -sporulation (■).

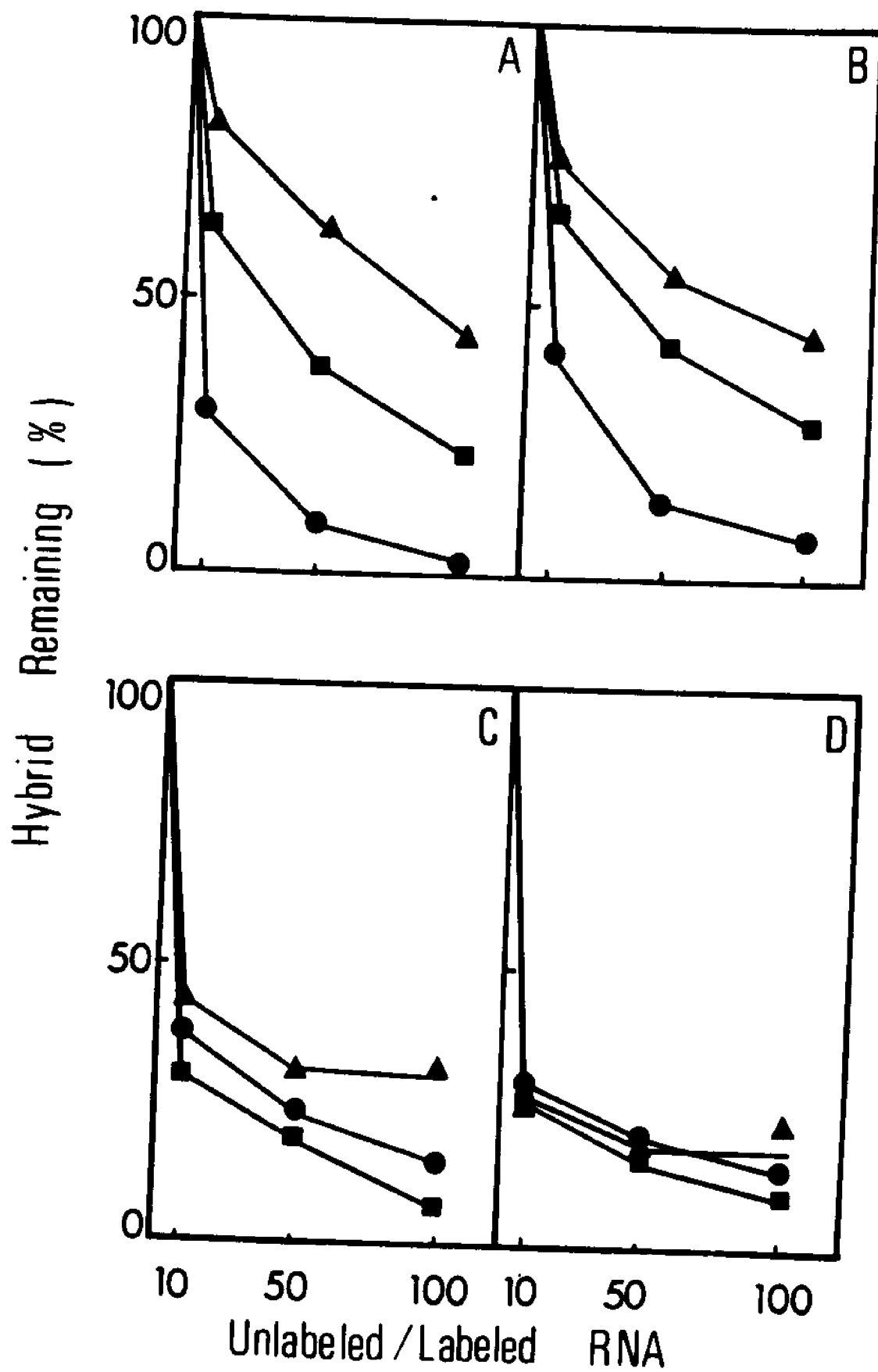


Figure 29 Competition of (^3H)labeled vegetative RNA with unlabeled vegetative and T_2 RNAs at high RNA/DNA ratios. Labeled RNA isolated from Bacillus subtilis strain A26u⁻ during vegetative growth in the Sonenshein and Roscoe Sporulation medium was annealed to individual DNA strands at a RNA/DNA ratio of 100:1 in the presence of increasing amounts of unlabeled competitor RNA. Each annealing mixture contained 0.2 μg of L or H DNA, 20 μg (^3H)labeled RNA (15,780 cpm/ μg) and increasing amounts of unlabeled bulk homologous and heterologous RNA in a final salt concentration of 6xSSC and in a total volume of 0.075 ml. This is the same RNA preparation as presented in Figure 27. (A) H strand DNA hybridizations (B) L strand DNA hybridizations. The 100% hybrid as cpm, in the absence of unlabeled competitor RNA, was 12,160 and 2,106 for H and L DNA respectively. Symbols for the unlabeled competitor RNA: Log (●), T_2 (▲) and $\text{T}_2\text{-CM}$ (□).

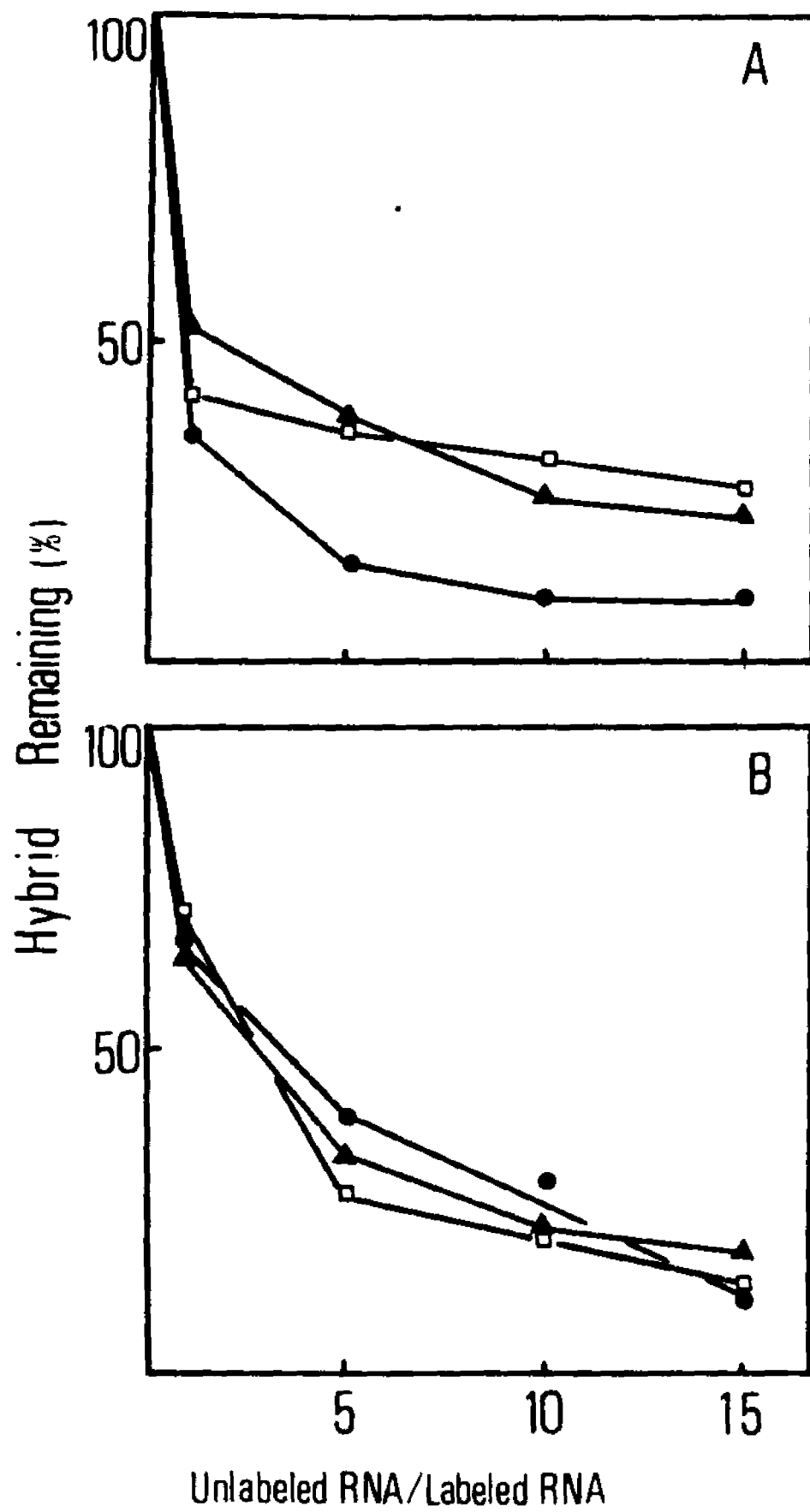


Figure 30 Competition of (^3H) labeled T_2 stationary phase RNA with unlabeled vegetative and T_2 RNAs at high RNA/DNA ratios. Labeled RNA isolated from Bacillus subtilis strain A26u⁻ during T_2 of stationary phase in the Sonenshein and Roscoe Sporulation medium was annealed to individual DNA strands at a RNA/DNA of 100:1 in the presence of increasing amounts of unlabeled competitor RNA. H DNA, 60 μg of (^3H) labeled T_2 RNA 100% hybrid as cpm, in the absence of unlabeled competitor RNA, was 4,817 and 1,071 for H and L DNA respectively and increasing amounts of unlabeled bulk homologous and heterologous RNA in a final salt concentration of 6xSSC in a total volume of 0.15 ml. This is the same RNA preparation as presented in Figure 27.

(A) H strand DNA hybridizations. (B) L strand DNA hybridizations.

100% hybrid as cpm, in the absence of unlabeled competitor RNA, was 4,817 and 1,071 for H and L DNA respectively. Symbols for the unlabeled competitor RNA: Log (●), T_2 (▲) and T_2 -CM (□).

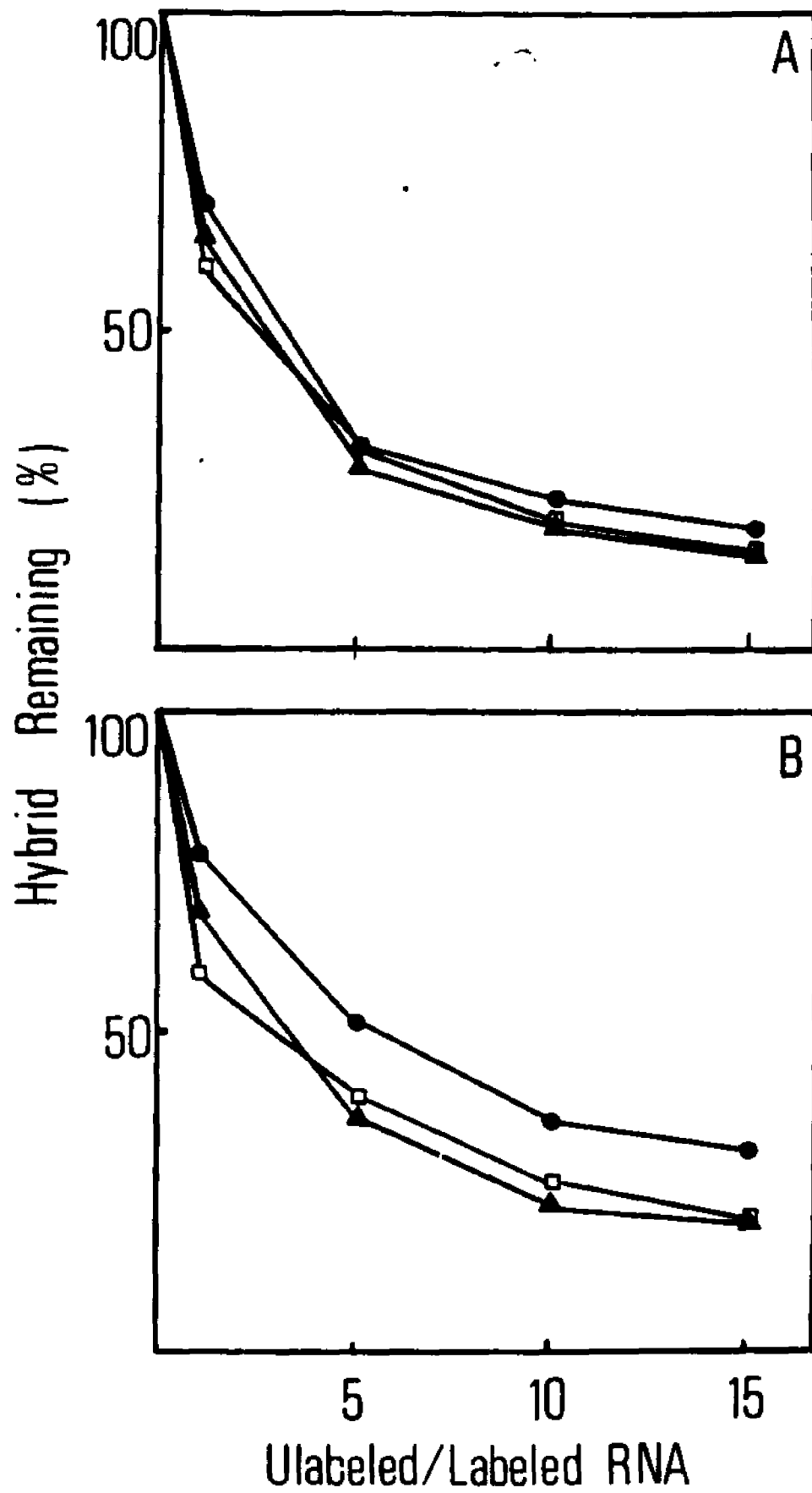


Figure 31 Competition of (^3H)labeled T_2 chloramphenicol-treated stationary phase RNA with unlabeled vegetative and T_2 RNAs at high RNA to DNA ratios. Labeled RNA isolated from Bacillus subtilis strain A26u⁻ treated during stationary phase with chloramphenicol (CM) in the Sonenshein and Roscoe Sporulation medium. Each annealing mixture contained 1.0 μg of L or H DNA, 100 μg of (^3H)labeled T_2 -CM RNA (specific activity, as cpm/ μg , 31,040) and increasing amounts of unlabeled bulk homologous and heterologous RNA in a final salt concentration of 6xSSC in a total volume of 0.28 ml. (A) H strand DNA hybridizations. (B) L strand DNA hybridizations. 100% hybrid as cpm, in the absence of unlabeled competitor RNA, was 5,488 and 1,290 for H and L DNA respectively. Symbols for the unlabeled competitor RNA: Log (●), T_2 (▲), and T_2 -CM (□).

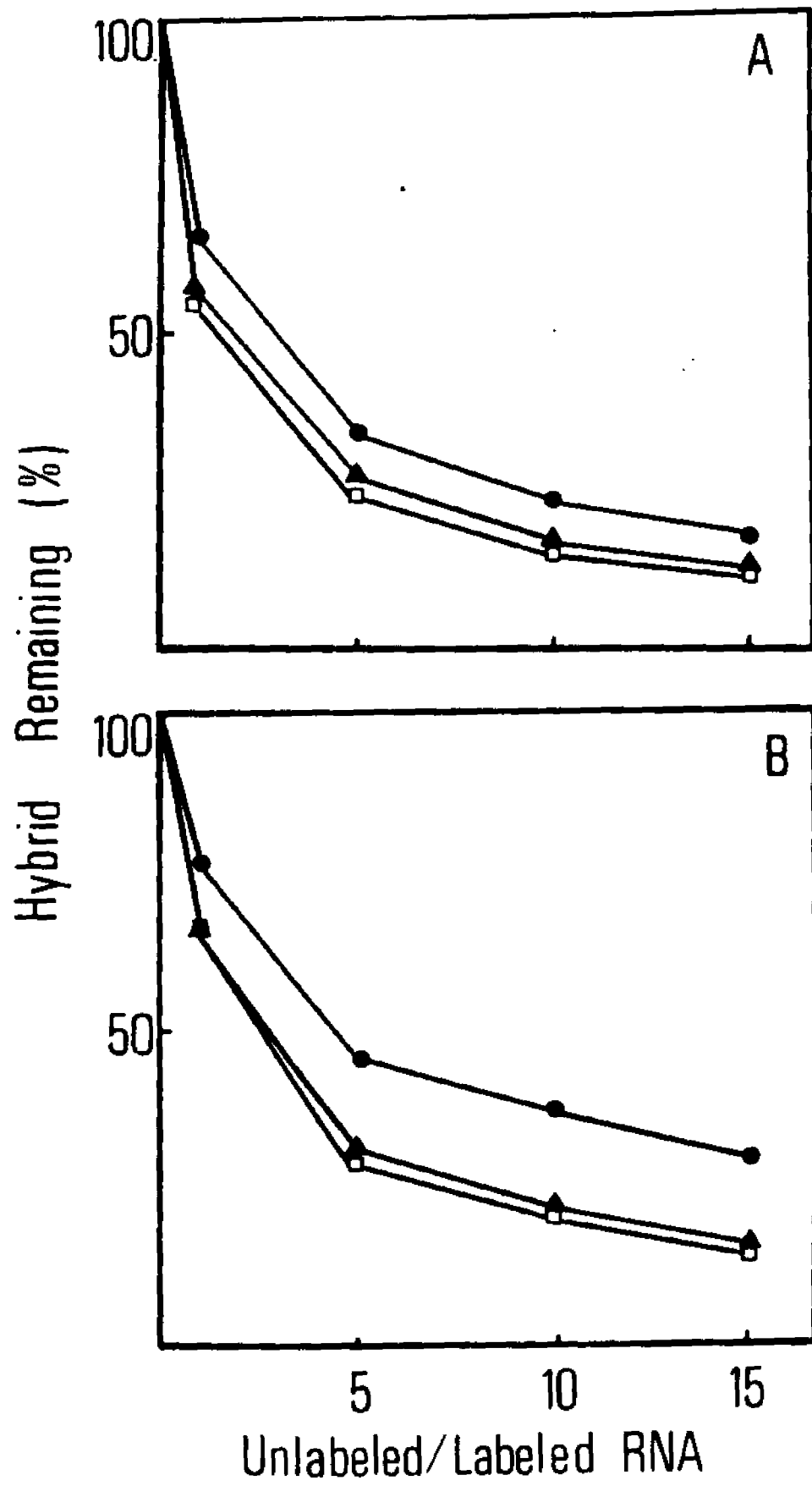


Figure 32 Summary of the hybridization-competition experiments for RNA extracted from sporulating (sp^+) and non-sporulating ($SpoM_c$) cells of Bacillus subtilis strain A26u⁻ grown in different sporulation media. One hundred percent competition is noted for each homologous RNA competition experiment. The relative percentage of competition has been calculated from the difference between the homologous and heterologous competition values in figures 24, 25, 26 and 31. (A), (B) and (C) are the panels for vegetative (^3H)RNA, sporulation T_2 double-labeled (^3H , ^{32}P) - RNA and sporulation T_4 double-labeled (^3H , ^{32}P) - RNA isolated from cells grown in SM-R media and hybridized to DNA at a RNA/DNA ratio of 10:1. (D), (E) and (F) are the panels for (^3H)labeled RNA preparations isolated from cells growing in 121B medium and hybridized to DNA at a RNA/DNA ratio of 100:1. These stages are respectively: vegetative RNA, T_2 stationary phase RNA, and T_2 stationary phase RNA made in the presence of chloramphenicol (CM). The open bars represent the H-DNA strand competition results and the closed bars represent the L-DNA strand competition results. The * indicates the radioactive RNA preparation. The notations in the abscissa represent the unlabeled bulk RNA which was used in the competition experiments.

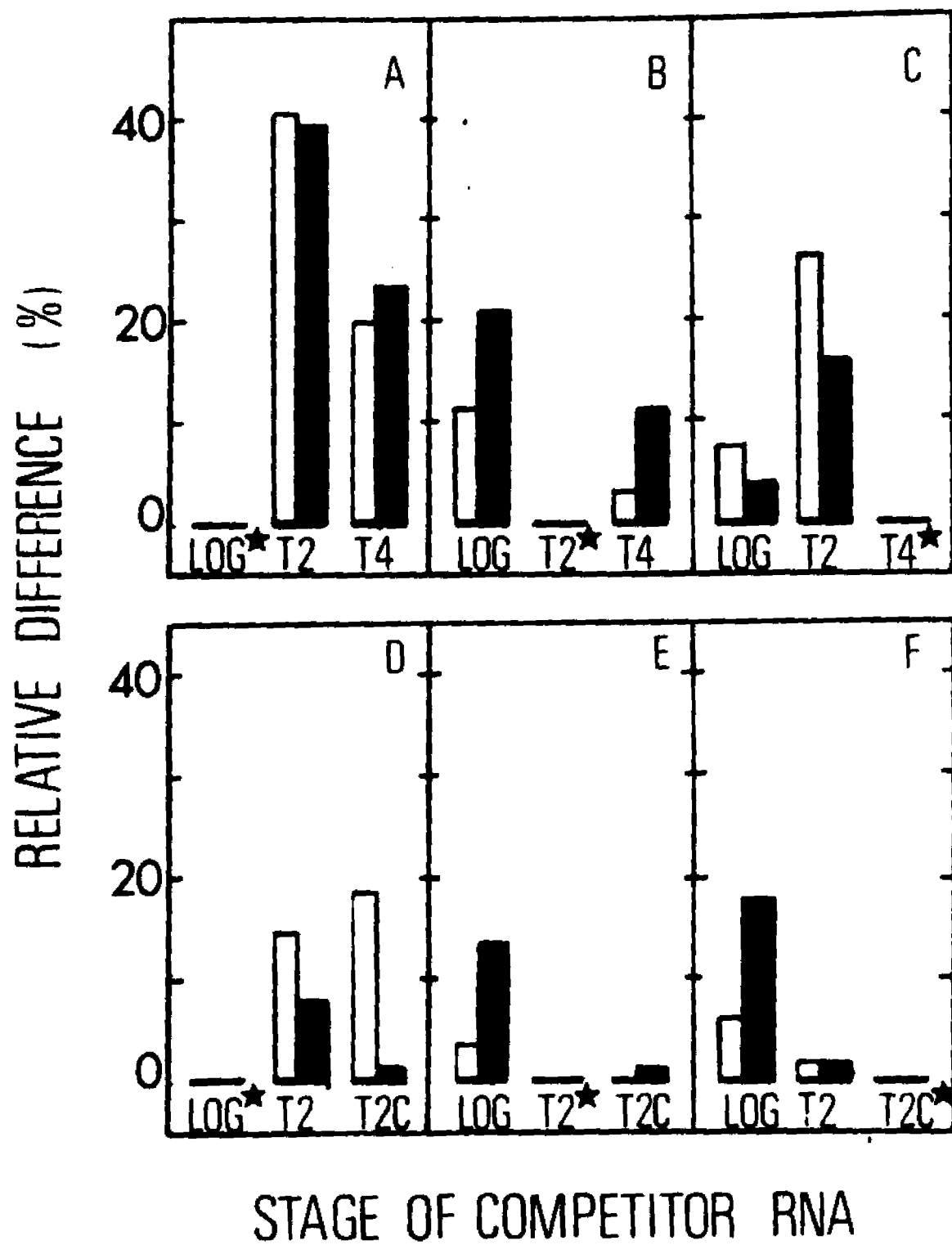
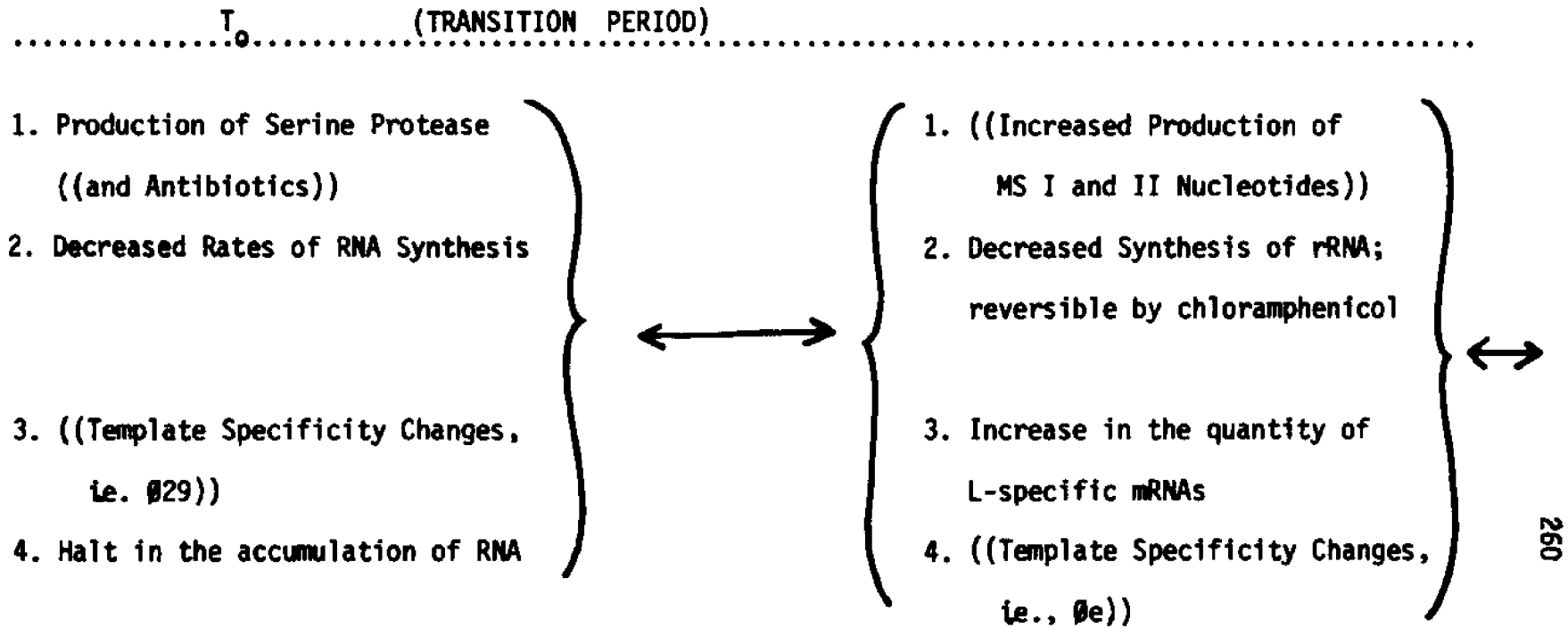
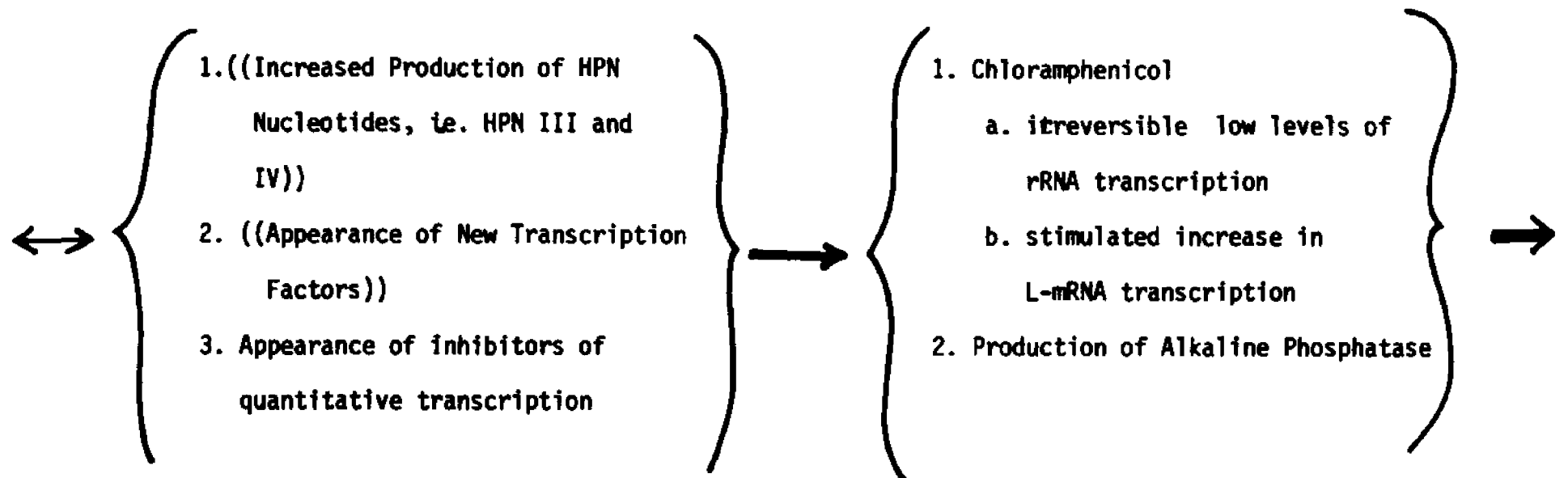


Figure 33

Scheme of Events Occurring During the Transition Period



.....INITIATION...PERIOD.....



(()) = Events not measured in these studies but reported elsewhere.