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Bacterial Spore Outgrowth: Its Regulation

During outgrowth, transcription is ordered.

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Although an ordered series of changes is observed during development or differentiation in a number of biological systems, there is little understanding of the control mechanisms involved. One promising system for exploring such changes is the conversion of a dormant spore into a vegetative cell (Fig. 1).

Under appropriate conditions, a population of activated spores will change from a dormant to a metabolically active state within a few minutes. This process is called "germination." "Outgrowth," the development of a vegetative cell from a germinated spore, occurs when germination takes place in a medium capable of supporting vegetative growth. Although outgrowth of a spore may last for several hours, it occurs synchronously within a population of spores. Periodic enzyme synthesis is observed during outgrowth, and several cycles of synchronous vegetative growth may follow it. The availability of a synchronous system makes it possible to carry out biochemical studies of metabolic events which are occurring in a timed sequence.

Initiation of Outgrowth

The details of techniques for activating spores and initiating germination have been extensively reviewed (*1*, *2*); some important points are pertinent here. Activation of dormant spores is usually achieved by heat treatment (for example, treatment at 65°C for 2 hours). This activation process appears to be reversible (*3*), but it is not well understood. Germination, on the other hand, is an irreversible and degradative process (*4*) which is not affected by metabolic inhibitors (*5*). During germination many unique properties of spores (*1*), such as refractility and resistance to heat, x-rays, and chemicals, are lost. This loss is accompanied by release of large amounts of spore-specific components. Germination is initiated by the addition of a germinating stimulant, or stimulants, to activated spores. These include amino acids (frequently L-alanine), nucleosides, and glucose (*1*). The degree of synchrony during outgrowth is dependent on the rate of germination, which in turn is dependent on the particular organism, the method of preparation of spores, the activation treatment, the medium in which germination takes place, and other physiological conditions (*p*H, temperature, buffer, and so on). In one of the most rapidly germinating species, *Bacillus cereus* T., addition of L-alanine initiates within 2 minutes, at 30°C, those morphological changes that are associated with germination (Fig. 2). After 6 minutes the changes are essentially complete. On the average, the time required for a single *B. cereus* T. spore to germinate is only 235 seconds (*6*). However, not all spores begin to germinate at the same time, and the germination kinetics of *single* spores may be complex (*7*, *8*). Thus, while germination is a rapid event for an individual spore, the germination time for a population is dependent on the span of time during which individuals begin the process. In cases of slowly germinating populations, this span may be an hour or more.

The fate of a germinated spore is largely determined by the availability of nutrients. A small amino acid pool in spores (*9*), as well as breakdown of mucopeptides (*10*) and possibly proteins, can provide a limited supply of amino acids for protein synthesis during outgrowth. These supplies are insufficient, and both prototrophic and auxotrophic strains require the addition of amino acids to support outgrowth (*11*). The time required to proceed through outgrowth to the first cell division is dependent upon the composition of the medium (Table 1). If the germinating medium is insufficient to support vegetative growth, either (i) development is arrested (*12*) or (ii) the outgrowing cell may proceed to form a second spore without intervening cell division (a process called "microcycle") (*13*). Microcycle is caused by conditions supporting sporulation, and the spores produced possess most of the properties of normal spores except for the ability to proceed through microcycle (*13*, *14*).

Order of Macromolecule Synthesis during Outgrowth

The morphological stages which occur during outgrowth have been described for several species of spores (*15*, *16*). Following germination, the spore swells, breaks out of its spore integument, and elongates.

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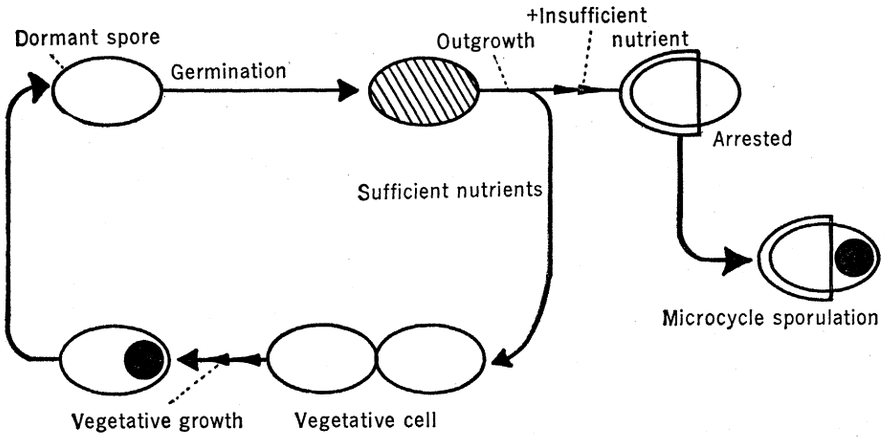


Fig. 1. Life cycle in *Bacillus*.

Biochemically, one can follow the various stages of outgrowth by examining the synthesis of RNA, protein, and DNA. Although the experimental data are complicated by variations in germinating and outgrowing populations, a general pattern in the order of macromolecular synthesis can be observed (Fig. 3). The synthesis of RNA is the first to begin after germination. This is closely followed by the onset of protein synthesis and by DNA synthesis at some later time (*11, 15, 17–20*). In *Bacillus cereus* T., RNA synthesis begins 2.5 minutes, and protein synthesis 4 minutes, after the addition of germination stimulants (*4*). Detectable net DNA synthesis does not begin until 120 minutes after the addition of stimulants, although incorporation of small amounts of radioactive thymidine is found as early as 5 minutes after the stimulants have been added (*21*). This incorporation represents synthesis of less than 10 percent of the total chromosome and has been identified in *B. cereus* T. and *B. subtilis* with DNA replication (*21, 22*).

In *Bacillus subtilis* the details of the kinetics of macromolecular synthesis are more complex, due to the differences in germination properties in various experiments. Armstrong and Sueoka (*19*), using spores which germinate within 5 minutes, found that RNA synthesis was biphasic. It began at 2 minutes after the addition of stimulants, increased linearly for 10 minutes, then leveled off for the next 8 to 10 minutes, when it again increased linearly. A linear rate of protein synthesis began at 15 minutes; this rate increased after 55 minutes. DNA synthesis was detected after about 45 minutes. Using spores which germinated more slowly (germination was complete at 100 minutes), Sakakibara *et al.* (*18*) found that RNA synthesis did not occur until 40 minutes after the addition of germinating stimulants. Protein synthesis began at 60 minutes, and DNA synthesis at 90 minutes. Thus, the basic pattern of the sequence of synthesis of RNA, protein, and DNA is conserved irrespective of the germination time (*23*).

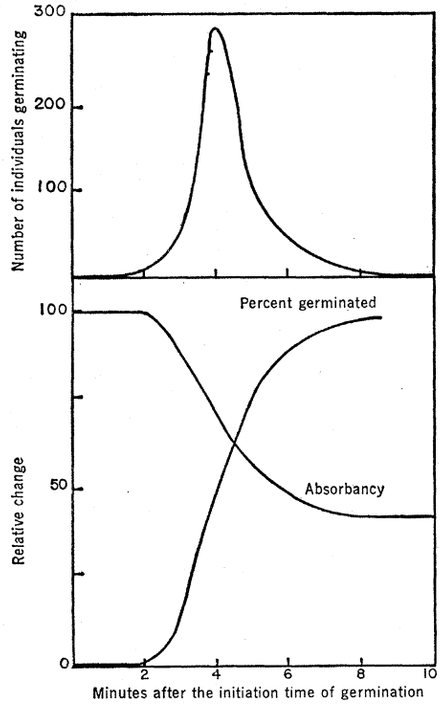


Fig. 2. Germination kinetics for a population of spores of *Bacillus cereus* T. The lower graph shows the increase in the percentage of the population germinated after germination stimulants had been added to a culture of activated spores, and the accompanying 40-percent decrease in the absorbancy of the culture. The upper graph gives a distribution of the number of individuals undergoing germination at any time. [Data from (*6*)]

Relation between RNA Synthesis and Protein Synthesis

The critical question in cryptobiotic systems is whether they contain packaged messenger RNA which can support protein synthesis upon reinitiation of growth. Protein synthesis during outgrowth is dependent on prior RNA synthesis. Actinomycin D, which has been shown to block DNA-dependent RNA synthesis in *Bacillus* (*24*), does not inhibit germination when added at zero time (that is, at the time the stimulants are added) but completely stops both RNA and protein synthesis which are normally observed (*18, 20, 25*). These and other findings (*20, 26*) led us to conclude that dormant spores, as well as newly germinated spores, contain little or no functional messenger RNA. Some doubt has recently been cast on this conclusion by the isolation of polysomes and an active protein-synthesizing system from spores of *B. megaterium* which had been lysed by treatment with urea, mercaptoethanol, ethylenediaminetetraacetic acid, and lysozyme (*27*). However, it has been subsequently demonstrated that, under these conditions, germination (*28*) and active RNA synthesis occur *prior* to lysis (*29*).

The lack of stored messenger RNA requires that suitable systems for the initiation of RNA synthesis exist in the spore. Indeed, the very early appearance of newly synthesized RNA (*7*) and the ability of RNA synthesis to proceed in the presence of chloramphenicol (CM) (*18*) demonstrate that RNA-synthesizing systems are intact in dormant spores, and that the synthesis of RNA is not dependent on protein synthesis (Fig. 4). Addition of actinomycin D during outgrowth immediately stops RNA synthesis; cessation of protein synthesis follows after 3 to 5 minutes (*20, 30*). RNA which has been synthesized before the addition of actinomycin D contains a labile fraction which is degraded in 3 to 5 minutes (*20, 31*). This effect of actinomycin D on RNA and protein synthesis during outgrowth demonstrates not only that protein synthesis is dependent on prior RNA synthesis but also that there is no evidence of stable messenger RNA produced during outgrowth.

Armstrong and Sueoka examined the pattern of messenger RNA synthesis in *Bacillus subtilis*, using sucrose gradient analysis of RNA labeled with [^{3}H]uracil. These authors described a

Table 1. Influence of medium on outgrowth. Heat-activated spores were germinated in the medium indicated. The synthetic medium contains seven amino acids plus salts of inorganic ions. The time for first division was taken as the time at which the first septum was observed. [Data from (*7*)]

Medium	Time for first division (min)	Type of growth at 15 to 20 hours
Complex	75	Vegetative
Synthetic minimal + glucose	220–240	Vegetative
Synthetic minimal + histidine		>25% microcycle
Synthetic minimal + glutamate	350	Vegetative
Synthetic minimal + glucose + eight additional amino acids	110–120	Vegetative

biphasic pattern in which messenger RNA was not synthesized until 16 minutes after the initiation of germination, whereas ribosomal and transfer RNA were synthesized immediately after germination (*19*). Conversely, in *B. cereus* T. a significant fraction of the initially synthesized RNA is neither ribosomal nor transfer RNA (*7, 32*).

In *Bacillus subtilis*, maturation of ribosomes is slow. Several workers have demonstrated that ribosomal RNA is made during the first phase of germination but is incorporated into mature ribosomes only after 2 hours in germination medium (*19, 23, 33, 34*). Transfer RNA is also synthesized immediately after germination (*11, 15, 19, 31*), but there is some question as to the relative amounts of ribosomal and transfer RNA that are made. By column chromatography on methylated albumin kieselguhr, Donnellan *et al.* (*11*) found that the ratio of transfer RNA to ribosomal RNA was relatively constant during outgrowth of *B. subtilis* and then changed when the system was in the vegetative state. Balassa and Contesse (*31*), however, found the ratio to be fairly constant during outgrowth and similar to that in the vegetative state.

To establish a more definitive relationship between the rate of protein synthesis and the amounts of various types of RNA during the early stages of outgrowth of *B. cereus* T. (*7*), the rate of phenylalanine incorporation into protein was compared to the amounts of uracil incorporation into labile and stable RNA, as well as to increases in total ribosomal RNA. As shown in Table 2, only the ratio of the rate of protein synthesis to the amount of labile RNA present is constant during outgrowth. Further, the capacity for protein synthesis as measured by amino acid incorporation after inhibition with actinomycin D closely parallels the kinetics of the synthesis of labile RNA (presumably messenger RNA) (*20, 30*). Germination thus leads to an interesting situation: a metabolically active state, containing functional ribosomes and a protein synthesizing system (*26, 27, 35*) but devoid of functional messenger RNA. At least during the initial phases of outgrowth, protein synthesis is transcriptionally controlled. Further evidence for this conclusion is presented in the next section.

Proteins and Enzymes Synthesized at Different Times during Outgrowth

Although the rate of protein synthesis immediately after germination appears to be dependent on the rate of messenger RNA synthesis, it remains to be shown what factors are important for determining in what sequence proteins are synthesized. That a definite sequence exists was initially observed by Kobayashi *et al.* (*20*), who found that, following germination, relatively few proteins were synthesized (Fig. 5). Torriani *et al.* (*12*) observed that later in outgrowth the pattern was more complex. The view that expression of the genome during outgrowth is nonrandom is supported by comparison of the pattern of incorporation of amino acids at two intervals of outgrowth.

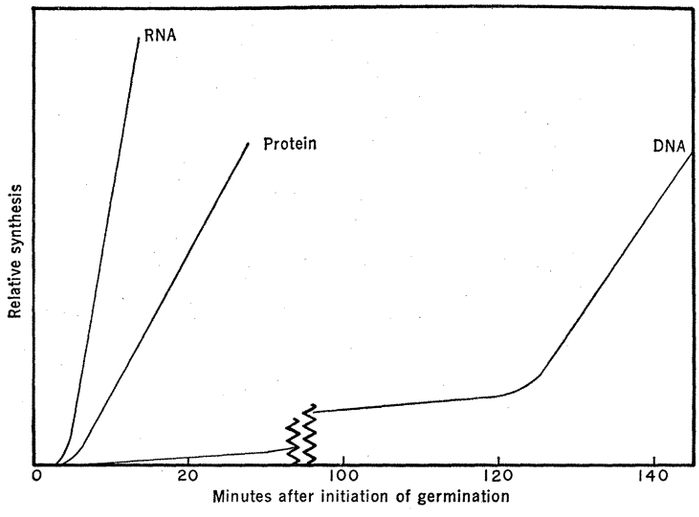


Fig. 3 (above). Synthesis of macromolecules following germination of spores. [Data from (*7*) and (*21*)]

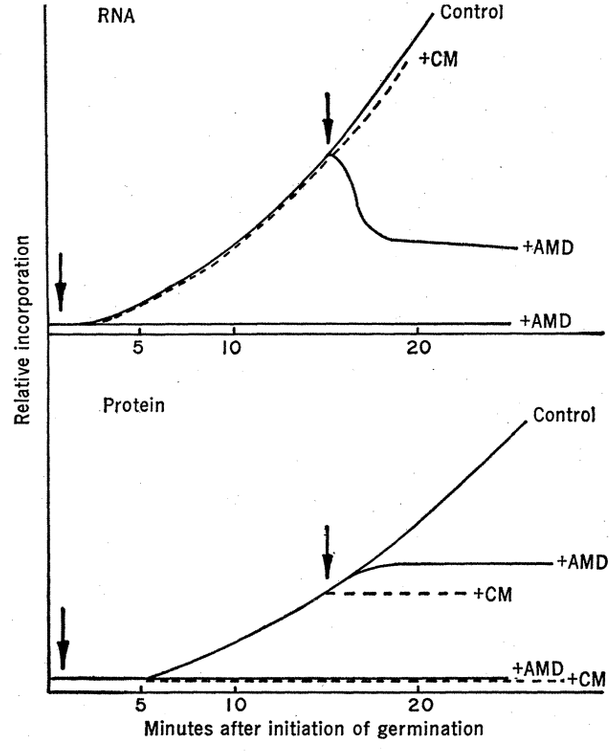
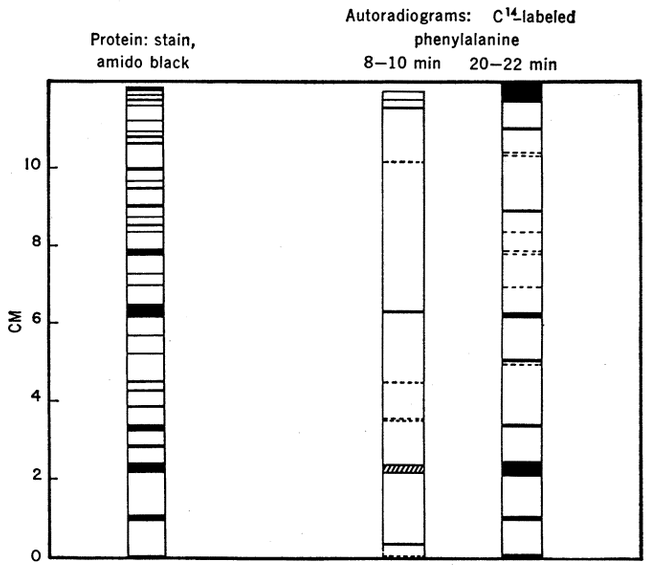


Fig. 4 (right). Inhibition of RNA and protein synthesis during early outgrowth. Incorporation of C^{14}-labeled guanine or uracil into RNA (upper graph) or of C^{14}-labeled amino acids into protein (lower graph) after the addition of chloramphenicol (*CM*) and actinomycin D (*AMD*) at 0 and 15 minutes (arrows) is shown as a function of time after the initiation of germination. [Data from (*18*), (*20*), and (*31*)]



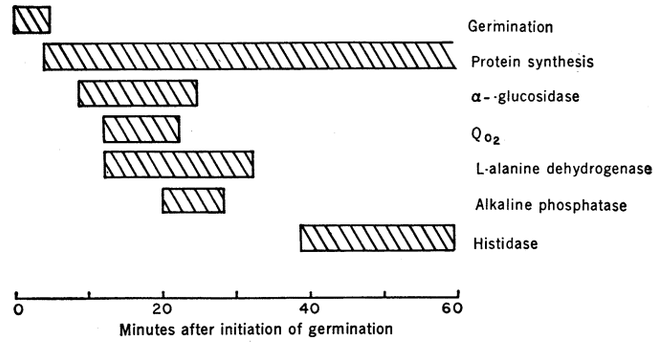


Fig. 5 (left). Labeling of classes of proteins during outgrowth. Total protein was isolated from spores which had been labeled with [^{14}C]phenylalanine for 2 minutes at the times indicated (in minutes) after the initiation of outgrowth. Autoradiograms of electropherograms are shown at right. Total protein, stained with amido black, is shown at left. [Data from (*20*)] Fig. 6 (above). Timing of specific events during germination. The shaded bars represent the *duration* of germination or specific synthesis during outgrowth of spores of *Bacillus cereus* T. [Data recalculated from (*7*), (*37*), and (*39*)]

When extracts of cells labeled at 30 minutes of outgrowth with [^{14}C]phenylalanine and at 120 minutes with [^{3}H]-phenylalanine were fractionated [by means of Sephadex and DEAE (diethylaminoethylcellulose) chromatography], significant differences between the distribution of C^{14}-labeled and H^{3}-labeled proteins were observed (*36*). In another experiment involving autoradiographic analysis of electropherograms in columns of polyacrylamide gels, isolation of selected proteins from the gels showed that each follows a separate time course of synthesis.

Not only have these broad classes of proteins been studied, but Steinberg *et al.* (*30*, *37*) have examined the kinetics of synthesis of induced α-glucosidase, L-alanine dehydrogenase, alkaline phosphatase, and induced histidase during outgrowth. Figure 6 shows the duration of synthesis for these enzymes during outgrowth. Of importance is the fact that there is a specific period for each activity during outgrowth in which the rate of synthesis rises to a maximum, but (more significantly) this rate then drops to zero.

Periodicity in the appearance of enzyme activity could be due to (i) temporal control of translation (or activation), (ii) selective modulation by small molecules of transcription (*38*), or (iii) temporal transcription of the genome. The results of two experiments make the first two possibilities seem unlikely. (i) Chloramphenicol added at any time blocks further formation of enzyme, indicating that it is being synthesized de novo and not being released from some inactive form. (ii) Actinomycin D stops enzyme synthesis within a few minutes. The capacity to support enzyme synthesis after actinomycin D has been added precedes and parallels the increase in the appearance of enzyme by a time equivalent to the lifespan of messenger RNA in *Bacillus* (Fig. 7). These findings suggest that messenger RNA's for these enzymes are produced periodically during outgrowth. To determine whether these are due to selective enrichment of messenger RNA species by ordered control of transcription (by induction, repression, and so on), the induction capacities of α-glucosidase and histidase were examined during outgrowth (*37*). It was observed that the period of induction was restricted to the time intervals indicated in Fig. 6.

Table 2. Dependence of the rate of protein synthesis on RNA during outgrowth. [Data from (*7*)]

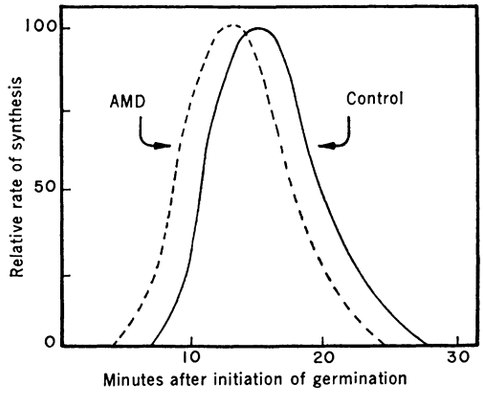
Minutes of outgrowth	Phenylalanine (ng) incorporated per minute per microgram of RNA*		
	Total RNA	Δ stable RNA	Labile RNA
10	0.12	5.86	9.20
20	.75	4.18	10.0
30	1.0	3.35	10.0
40	1.2	2.51	10.0
50	1.1	2.09	10.0
70	0.9	1.67	9.20
90	.8	0.84	9.20

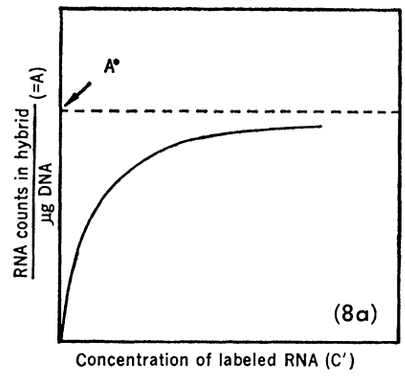
* The increase in stable RNA (Δ stable RNA) and amounts of labile RNA during outgrowth were calculated from a plot of incorporation of [^{14}C]uracil into RNA, either stable or labile, in the presence of actinomycin D. Total RNA was measured colorimetrically.

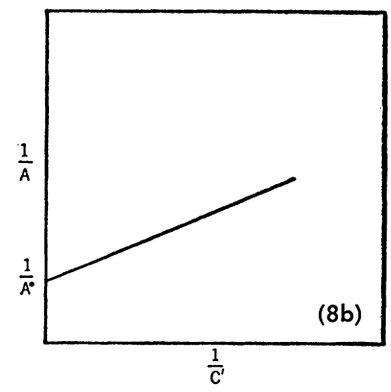
Messenger RNA Synthesized at Different Times during Outgrowth

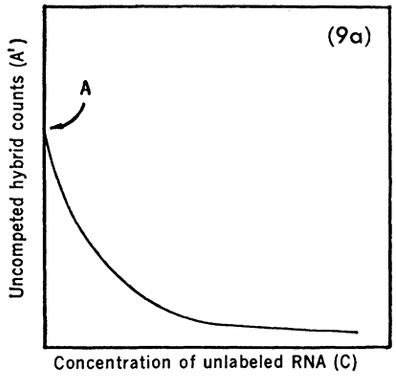
Although these results demonstrate that specific protein synthesis is a regulated phenomenon dependent upon the presence of specific messenger RNA, they do not definitely establish a transcriptional control. To directly demonstrate such control, it is necessary to compare the messenger RNA populations transcribed at different intervals during outgrowth. If few differences are found, this would suggest that most of the genome is continuously available for transcription. If, on the other hand, major differences are found between messenger RNA's synthesized at different intervals, then this would constitute evidence for control at the level of transcription.

Under appropriate restrictions, graphs of data obtained from RNA-DNA hybridization experiments involving competition of labeled and unlabeled species of RNA provide a method for comparing the predominant species of messenger RNA present at two intervals during outgrowth. Although hybridization of pure species of RNA









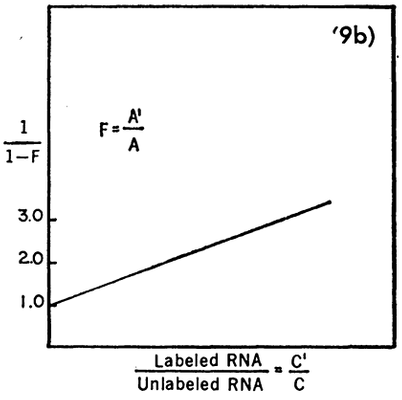


Fig. 7 (above). Timing of synthesis of α-glucosidase induced by messenger RNA. The control represents the relative rate of synthesis of α-glucosidase during outgrowth. At intervals, actinomycin D (10 micrograms per milliliter) was added, and enzyme concentration was assayed 37 minutes later. The dashed line represents the capacity to support enzyme synthesis after the addition of actinomycin D. [Data from (*37*)] Fig. 8 (above right). Theoretical saturation curve for a single homogeneous species. A is the number of RNA counts bound in the hybrid per microgram of DNA; C' is the concentration of radioactive RNA. (a) Theoretical saturation curve for hybridization of a single homogeneous species of radioactive RNA to DNA. A^* equals A when C' equals infinity. (b) A double-reciprocal plot of the curve shown in the graph at left, conforming to the equation $1/A = (K/A^*C) + (1/A^*)$. Fig. 9 (right). Theoretical competition curves of homogeneous labeled RNA and heterogeneous competing RNA. A is the number of counts of RNA bound per microgram of DNA in the absence of competing unlabeled RNA. A' is the number of counts of RNA bound in the presence of varying amounts of unlabeled RNA. C is the concentration of unlabeled RNA; C', of labeled RNA. (a) Theoretical competition in which a single homogeneous species of unlabeled RNA competes with an identical labeled RNA for homologous sites on the DNA. (b) A double-reciprocal plot of the curve shown in the graph at left; it conforms to the equation $1/(1-F) = (C'/C)\ (A^*/A) + 1$, where F is equal to A'/A.

with DNA immobilized on nitrocellulose membranes (*39*) conforms to simple isothermal kinetics, saturation curves involving mixtures of several RNA species are exceedingly complex. Competition curves of complex RNA mixtures are more easily interpreted. For purposes of illustration, it is useful to examine a theoretical saturation curve (isotherm) for a single homogeneous species of radioactively labeled RNA.

The graph of Fig. 8a is described by the equation

$$\frac{A}{A^*} = \frac{C'}{K + C'}$$

As the concentration (C') of labeled RNA increases, A, the number of RNA counts bound per microgram of DNA, increases to a maximum (A^*). K is a constant. This equation may be rearranged into a double-reciprocal form, shown in Fig. 8b.

$$\frac{1}{A} = \frac{1}{C'} \cdot \frac{K}{A^*} + \frac{1}{A^*}$$

Since this labeled RNA is composed of a single species, addition of unlabeled RNA of the same species will result in a simple isotopic dilution curve, shown in Fig. 9a. An equation may be written which accounts for isotopic dilution as well as for the change in the level of saturation caused by a different concentration of RNA.

$$\frac{1}{A'} = \frac{K}{(C + C')A^*\left(\frac{C'}{C' + C}\right)} + \frac{1}{A^*\left(\frac{C'}{C' + C}\right)}$$

If F is defined as A'/A, then FA may be substituted for A' in this equation. The significance of A'/A is indicated by Fig. 9a. Rearrangement then gives

$$\frac{1}{1-F} = \frac{C'}{C} \cdot \frac{A^*}{A} + 1$$

Figure 9b is a replot of the theoretical data from Fig. 9a with $1/(1-F)$ as the ordinate and C'/C as the abscissa. The curve is a straight line with a slope equal to the reciprocal of the fraction for saturation of the DNA by labeled RNA in the absence of competing RNA. A slope of 2 means that, in the competition study, radioactively labeled RNA was used in a concentration sufficient to half-saturate the corresponding sites of the DNA. The intercept of $1/(1-F) = 1$ means that, as the concentration of unlabeled RNA approaches infinity, F approaches zero, the point at which competition is complete.

If a heterogeneous RNA instead of a pure species is to be used in the competition experiment, the concentration of unlabeled RNA which is competing is

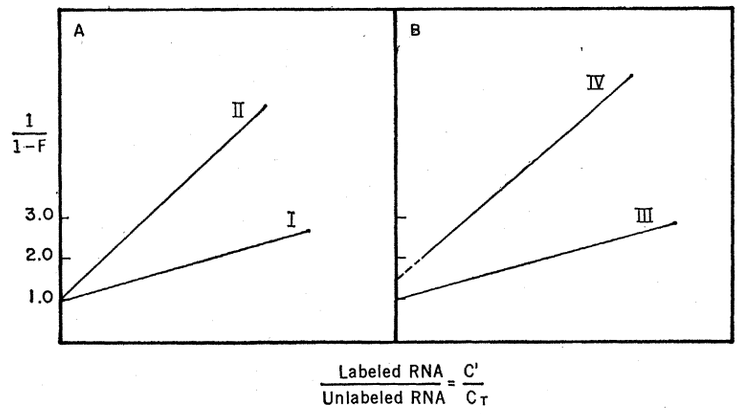
$$C_T \cdot \frac{C}{C_T}$$

where C_T is the total unlabeled RNA and C/C_T is the fraction of species which can compete. The above equation may be rewritten as

$$\frac{1}{1-F} = \frac{C'}{C_T} \cdot \frac{C_T}{C} \cdot \frac{A^*}{A} + 1$$

A plot of C'/C_T, or the ratio of labeled RNA to total unlabeled RNA, gives a straight line with a slope of

$$\frac{C_T}{C} \cdot \frac{A^*}{A}$$



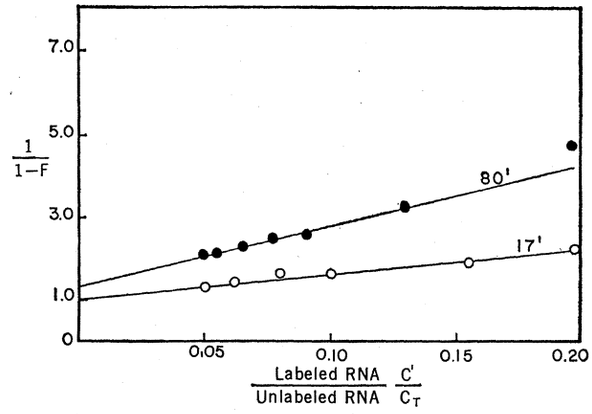


Fig. 10 (left). Theoretical competition curves for heterogeneous RNA. (A) A double-reciprocal plot of two competition curves. In both cases the labeled RNA is a single homogeneous species. RNA I (unlabeled) and the labeled RNA are homologous. RNA II (unlabeled) is heterogeneous and contains RNA I. (B) A double-reciprocal plot of two competition curves. In both cases the labeled RNA is heterogeneous, containing more than one species of labeled RNA. RNA III is unlabeled and *identical* to the labeled RNA preparation. RNA IV (unlabeled) contains some species of RNA not present in RNA III, and RNA III contains some species of RNA not present in RNA IV. Fig. 11 (right). Competition of 8-minute pulse-labeled RNA with 17- and 80-minute RNA. See (*48*) for details.

The presence of a noncompeting RNA thus increases the slope. Since this equation holds true for any species of RNA, it may be summed from species 1 to species i, where i is the number of competing species.

$$\sum_{1}^{i} \frac{1}{1-F} = \frac{C'}{C_T}\left[\sum_{1}^{i} \frac{C_T}{C} \cdot \frac{A^*}{A}\right] + 1$$

Division of both sides by i gives

$$\frac{1}{1-F} = \frac{C'}{C_T}(\text{average slope}) + 1$$

This is also a straight line with an intercept of 1. The slope is a measure of the heterogeneity of the competing species. This heterogeneity of labeled RNA changes the slope of the line but does not change the nature of the curve. Generally speaking, if two unlabeled RNA preparations are used as species competing against the same labeled RNA, the curve with the greater slope will be given by the unlabeled RNA, which is more heterogeneous than the labeled RNA. Theoretical examples of these types of curves are given in Fig. 10A. RNA I (see the legend to Fig. 10) has fewer noncompeting species than RNA II has; that is, closer homology is found between the labeled RNA and RNA I (unlabeled) than between the labeled RNA and RNA II (unlabeled).

It is easy to imagine that predominating species in the labeled RNA could occur as minor or nonexistent components of the competing RNA. For these components, even the highest experimentally attainable concentrations of competing RNA will give insufficient competition to allow accurate extrapolations. What will be obtained upon extrapolation is an apparent $1/1 - F$ intercept greater than 1, which will reflect the number of predominating species in the labeled RNA that are minor or nonexistent components of the unlabeled competing RNA. A theoretical example of this is shown in Fig. 10B. Curve IV does not intercept at $1/1 - F = 1$. This implies that there are radioactive species which are absent from RNA IV (or present only in low amounts) but present in RNA III.

Let us now consider the similarities between RNA populations synthesized at different intervals of spore outgrowth. Figure 11 shows competition curves for pulse-labeled RNA isolated 8 minutes after germination of *Bacillus cereus* T. spores. Competing unlabeled RNA was isolated after 17 and 80 minutes, respectively. The slope and intercept of the curve for 17-minute competing RNA indicate that there is considerable similarity between RNA synthesized at 8 minutes and RNA synthesized at 17 minutes. Conversely, 80-minute RNA has both a greater slope than 8-minute RNA and a $1/1 - F$ intercept greater than 1. When we solve for F, it seems that about 23 percent of the predominant radioactive species present in 8-minute RNA are absent from 80-minute RNA. Further, the slope indicates that those species present in 80-minute RNA which are also present in newly synthesized 8-minute RNA are in low concentration in 80-minute RNA. This study effectively demonstrated that the species of RNA being synthesized during these time intervals are not the same. The data of Table 3 further support this conclusion. Table 3 shows the results of competition experiments with labeled and unlabeled RNA species isolated at additional time intervals during outgrowth. The DNA papers used in these experiments were hybridized in a preliminary procedure ("prehybridized") with transfer and ribosomal RNA to eliminate ambiguities due to binding of stable species. Of all the combinations of RNA used, very close homology is

Table 3. Results from double-reciprocal plots of competition curves for RNA obtained at various time intervals during outgrowth.

Time of isolation (minutes after germination)		Slope*	Intercept*	F_{min}†	Hybridized‡
Unlabeled RNA	Labeled RNA				
17	8	6.0	1.0	0	Yes
40	8	Very large	Very large	~1	Yes
80	8	14	1.4	0.29	Yes
8	40	16	1.3	.23	No
80	40	5.5	1.2	.17	No
40	80	Very large	Very large	~1	Yes

*The slopes and intercepts were obtained from a plot of $1/(1 - F)$ relative to C'/C. † F_{min} is the fraction of labeled RNA which is unaffected by the addition of competing unlabeled RNA. It is calculated from the intercept where $C'/C = 0$. ‡Hybridizations were carried out as described in (*48*).

found only between 8-minute and 17-minute RNA. This finding is not unexpected, for the time points are rather close together. On the other hand, 40-minute unlabeled RNA competes very poorly with either 8-minute or 80-minute RNA. Therefore, it appears that predominant species being synthesized at 8 and 80 minutes are minor components of 40-minute RNA. Since preliminary hybridization has eliminated binding of stable species, the results represent comparisons only of unstable RNA species.

It is interesting to compare these results with those of experiments with labeled 40-minute RNA, with 8- and 80-minute RNA as competing species. In this case, about 80 percent of the RNA being synthesized at 40 minutes is present as prominent species in 8- and 80-minute RNA. This suggests that 8- and 80-minute RNA are similar. Indeed, Table 3 shows that about 70 percent of the newly synthesized 8-minute RNA is present as prominent species in 80-minute RNA. That some RNA synthesis during outgrowth is under temporal control is evidenced by the finding that one (or more) class of unstable RNA is transcribed prominently at 8 minutes and at 80 minutes but not at 40 minutes.

Concluding Remarks

Germination of a bacterial spore in a suitable medium leads to a developmental process (outgrowth) which, at least in its early stages, is characterized by specific patterns of protein and enzyme synthesis. As discussed here and elsewhere (*33, 40*), these patterns are dependent upon prior RNA synthesis. During the early stages of outgrowth, ribosomes do not limit the rate of protein synthesis. The correlation between (i) synthesis of individual enzymes during early outgrowth, with their limited period of inducibility (*37*) and (ii) the capacity of the outgrowing spore to synthesize enzymes in the *absence* of further RNA synthesis (Fig. 7) argues for regulation at the level of transcription. One prediction of this conclusion is that the populations of *prominent* messenger RNA species should fluctuate during early outgrowth. In this article we describe a methodology for comparing the prominent species of rapidly labeled RNA present at two different developmental stages. Within the limitations listed below, it is possible, for the first time, to conclude that the distribution of unstable, rapidly labeled RNA species (assumed to be messenger RNA) fluctuates during early outgrowth; that is, transcription is regulated.

While it is generally recognized that DNA–RNA hybridization has proved valuable for examining questions involving stable (*39*) or other homogeneous RNA's (*41, 42*), such hybridization has been less useful for studying complex mixtures of messenger RNA. Consideration of competition curves involving a mixture of predominant and minor species leads to the conclusion that these curves reflect only the presence of quantitatively predominant RNA species and cannot reflect the presence of minor species (*41*).

Minor species of RNA will not hybridize significantly unless exceedingly high RNA concentrations (frequently approaching the maximum solubility of RNA) are used. Competition experiments with even larger amounts of unlabeled RNA are impossible. Therefore, in competition experiments one must use concentrations of labeled RNA at which its minor component species are hybridized poorly. There is a similar limitation when the competing RNA has minor components which do not compete effectively at experimentally attainable concentrations.

At the relatively low concentrations of radioactively labeled RNA (about 5 percent of RNA solubility) used in these competition experiments, only predominating radioactive species are present in sufficient concentration to approach saturation when competing RNA is added. It is important to realize, therefore, that only predominant species are reflected in a hybridization-competition curve. One cannot distinguish between the presence of a minor species and its absence. The inability to detect minor species of RNA in competition experiments in which rapidly labeled RNA (messenger RNA) is used limits very importantly the conclusions one can draw about homogeneity between two RNA populations. If competition between two RNA preparations is incomplete, only quantitative differences have been demonstrated. In spite of these limitations, competition is a very useful tool in detecting differences between RNA populations. The graphical method presented here for plotting competition data gives rise to a straight line, whose slope and intercept are meaningful in terms of heterogeneity. Specifically, the slope of the line is directly proportional to the degree of heterogeneity. Also, the intercept is a measure of the fraction of labeled RNA containing predominant species which are either minor components of the competing RNA or are altogether absent.

Outgrowth of the bacterial spore, like other developmental systems, is undoubtedly under a wide variety of controls. Some of these may be unique to the spore state itself (*12*). Numerous models have been proposed to explain the periodicity frequently characteristic of developmental systems (for a review, see *43*). The possibility that this type of control is responsible for the periodicity in enzyme synthesis during outgrowth of spores of *Bacillus cereus* T. (*21*) as well as in the case of autogenous enzyme synthesis in vegetative cells of *B. subtilis* (*44*) can be ruled out. In both cases, periodic enzyme synthesis continues in the absence of DNA replication.

One observation that must be considered in designing models for outgrowth is that transcriptional controls are not uniform throughout the genome. As calculated by Steinberg *et al.* (*45*) and demonstrated experimentally (*11, 12, 23, 32, 33, 46*), the ribosomal cistrons are transcribed continuously during outgrowth although they constitute a minor fraction of the genome (*32*). The evidence presented in this article shows that individual species of rapidly labeled RNA are synthesized periodically. The evidence of periodicity is consistent both with the possibility that there are repeated rounds of transcription of all messenger RNA genes (*47*) and with the possibility that there is a transition, during outgrowth, from genes transcribed early to others transcribed only at later times (*12*).

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Stable Carbonium Ions in Solution

New superacid solvents and nuclear magnetic resonance spectroscopy permit direct study.

George A. Olah

In organic chemical reactions, reactive intermediates like carbonium ions, carbanions, free radicals, radical ions, carbenes, nitrenes, and arynes were for a long time considered only as transient, metastable species with very short lives. Rapid advances in experimental and spectroscopic techniques have made an increasing number of reactive intermediates available for direct observation and study (*1*). One of the most important classes of reactive organic intermediates is that of carbonium ions.

Early Studies

Less than a year after the discovery of the first stable radical, the triphenylmethyl radical, by Gomberg in 1900, Norris (*2*) in the United States and Kehrmann and Wentzel (*3*) in Germany observed that colorless derivatives of triphenylmethane, such as triphenylmethyl alcohol or chloride, give deeply colored solutions when dissolved in sulfuric acid. Similarly, triphenylmethyl chloride forms colored complexes with aluminum and tin chlorides. In 1902 von Baeyer (*4*) recognized the salt character of these compounds and named them carbonium salts.

As mentioned, the observation of the first stable carbonium ions was achieved with triphenylmethyl systems some 70 years ago. It is somewhat surprising that, despite the substantial amount of data gathered in this specific field (as a result of widespread interest in connection with dye chemistry), carbonium ion chemistry was long considered by organic chemists as a topic limited to triarylmethyl dyes. No attempt was made to extend the scope to other areas until nearly 20 years later. The analogy between the development of carbonium ion chemistry and of free radical chemistry is evident. It is also apparent that the availability of newer physical methods, like electron spin resonance spectroscopy in the free radical field and nuclear magnetic resonance spectroscopy in the carbonium ion field, helped substantially to extend our knowledge. In a brief review only selected areas of the large field can be discussed. The selection reflects my own interest as an organic chemist and in addition covers some aspects of biological interest, which may be of future importance.

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