*MESSENGER RNA TURNOVER AND PROTEIN SYNTHESIS IN B. SUBTILIS INHIBITED BY ACTINOMYCIN D**

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The hypothesis that protein synthesis in bacteria is mediated by an unstable, rapidly turning over RNA fraction, designated messenger RNA (mRNA),[1] is well supported by evidence from a variety of experiments. When either phage-infected[2-4] or normal[5, 6] bacterial cells are given short pulses of radioactive RNA precursors, the radioactivity is incorporated into an unstable RNA having a base composition similar to that of DNA and able to form molecular hybrids with it.[7] This RNA fraction is distinguishable from the ribosomal and transfer RNA by its sedimentation behavior and by its ability to stimulate protein synthesis in cell-free ribosomal systems.[8, 9]

Experimental evidence as to the fate of mRNA molecules has been meager however and is subject to various interpretations.[10] Furthermore, the number of times a given mRNA molecule functions during the formation of protein has not been determined. The elucidation of both of these problems depends upon preventing the reutilization of any breakdown products of mRNA either to form more messenger or to form stable RNA. A substance which might be suitable for this purpose is actinomycin D, an inhibitor of RNA synthesis.[11] Actinomycin D is known to attach to DNA[12] and to prevent RNA synthesis in *Staphylococcus*, *Neurospora*,[13] mammalian cells, and in cell-free systems.[11, 14, 27] It allows formation of RNA viruses in mammalian cells, but not of DNA viruses.[15, 27] It seems likely, therefore, that it inhibits specifically DNA-dependent RNA formation.[14, 27]

The results of our investigation of the fate of mRNA and the course of protein synthesis in cells of *B. subtilis* in which RNA synthesis was inhibited by actinomycin indicate that a single mRNA molecule is utilized several times in protein synthesis and that it breaks down with a time constant of about 2 min into acid-soluble, low molecular weight material.

Material and Methods.—*B. subtilis* strain W-23 (described in ref. 16) was grown in a Tris-glucose medium supplemented with tryptone at a concentration of 0.1 mg/ml. Overnight cultures were diluted, incubated for 2 generations at 33.5°C, and used during the logarithmic growth phase at an optical density between 0.3 and 0.35, measured at 540 mμ wavelength on a Zeiss spectrophotometer. The doubling time in this medium was approximately 70 min (generation time 100 min). Actinomycin D (Merck, Sharp and Dohme) was dissolved in 70% alcohol at a concentration of 1.0 mg/ml and stored in the dark at 0°C. All experiments were carried out in dim light. Chloramphenicol was a Parke Davis product; uniformly labeled C^{14}-valine and phenylalanine, and C^{14}-uracil labeled in the 2 position, were obtained from the New England Nuclear Corporation.

Sampling and counting procedures: C^{14}-uracil, incorporated into high molecular weight RNA, was measured by rapidly diluting the labeled culture into an equal volume of 10% TCA at 2°C containing 100 μg uracil per ml. After storage in the cold for at least 30 min the precipitate was collected on membrane filters (0.45 micron pore size, from Millipore, Inc.). The filters were presoaked in 5% TCA plus uracil, and the samples were washed on the filter with 5 washings of 3 ml each of 5% TCA-uracil. The filters were then glued onto counting planchets with rubber cement, dried, and counted in an end window Geiger counter.

For measuring incorporation of C^{14} amino acids into protein, metabolic processes were stopped by dilution of 2 ml of the culture into an equal volume of cold 10% TCA containing 1.0% cas-

amino acids. After storage for at least 30 min, the samples were centrifuged and the pellets were resuspended in 1.5 ml of 1 *M* NaOH and held at room temperature for 20 min. 6 ml 10% TCA with casamino acids were then added, the samples were heated to 95°C for 30 min, and the precipitates were collected on membrane filters, washed and plated as described above. All samples were counted for at least 2000 counts.

Sedimentation analysis of labeled RNA was carried out using the sucrose gradient technique described by Britten and Roberts.[17] The labeled cells were added to crushed ice, centrifuged, and washed with Tris buffer, 0.01 *M*, pH 7.4, containing $10^{-4}M$ Mg^{++}. An alumina extract was prepared and layered on a sucrose gradient. All operations were carried out at 2°C. After centrifugation for 6 hr at 24,000 rpm in a Spinco SW 25 rotor centrifuge, the tube was punctured and fractions were collected for measurement of TCA precipitable C^{14} and optical density at 260 mμ.

The inhibition of growth of *B. subtilis* by actinomycin D was examined at various concentrations of the antibiotic. The results indicated that 0.5 μg/ml reduced the growth rate by approximately 50%, while at concentrations of 1.0 μg/ml and above there was no appreciable increase in optical density after the antibiotic was added to the culture. All experiments were carried out using a concentration of 10 μg/ml. Control experiments indicated that the alcohol in which the antibiotic was dissolved did not alter the growth rate or the uptake of C^{14}-valine or C^{14}-uracil.

Results.—Experiment 1: In order to determine the time required for actinomycin to inhibit RNA synthesis, the antibiotic and C^{14}-uracil were added simultaneously to a culture of *B. subtilis*. It is evident from Figure 1 (curve *c*) that the uptake of C^{14}-uracil was almost immediately and totally suppressed. Cells incubated in the presence of C^{14}-uracil and actinomycin D for 60 min incorporated less C^{14} than did those in the control culture, without actinomycin, in 15 sec. The uptake of uracil in the control was linear from the time of the addition of C^{14}-uracil, indicating that the pool of uracil within the cell was less than about 1% of the total uracil in RNA.

Experiment 2: Having found that actinomycin stops all new RNA synthesis in *B. subtilis*, we wished to determine the fate of mRNA synthesized immediately before the addition of the antibiotic. Cells were preincubated for various lengths of time in C^{14}-uracil. Actinomycin was then added and samples were removed and rapidly added to cold TCA. Under these conditions some of the radioactivity which had been incorporated into RNA prior to the addition of actinomycin became acid-soluble on further incubation in the presence of the antibiotic. In Figure 2 the fraction of the C^{14} remaining acid-precipitable is given as a function of the time after the addition of actinomycin. Increasing the time of preincubation with C^{14}-uracil decreases the percentage of label which eventually becomes acid-soluble. That portion of the C^{14} that decays does so with approximately first-order kinetics, having a mean decay time of about 2 minutes. In Figure 1, curve *b*, the amount of label in stable RNA (that is, the fraction that does not decay in actinomycin) is given as a function of time of preincubation before the addition of actinomycin.

Experiment 3: The sedimentation pattern of *B. subtilis* mRNA was determined by giving a 30-sec pulse of C^{14}-uracil to cells growing with a generation time of 100 min (Fig. 3). This pattern is similar to those obtained with *E. coli* in short pulse experiments.[18] The UV absorption pattern shows the 50*S* and 30*S* ribosomes and the soluble RNA at about 4*S*. The RNA labeled with C^{14} in 30 seconds has sedimentation properties similar to the messenger fractions obtained in *E. coli*. There is a peak at approximately 16*S* as well as a considerable amount of heavier material.

Experiment 4: In order to determine whether the RNA which decays in the presence of actinomycin has the sedimentation properties of messenger, cells were labeled with C^{14}-uracil for 3 minutes. This time of preincubation is sufficient for

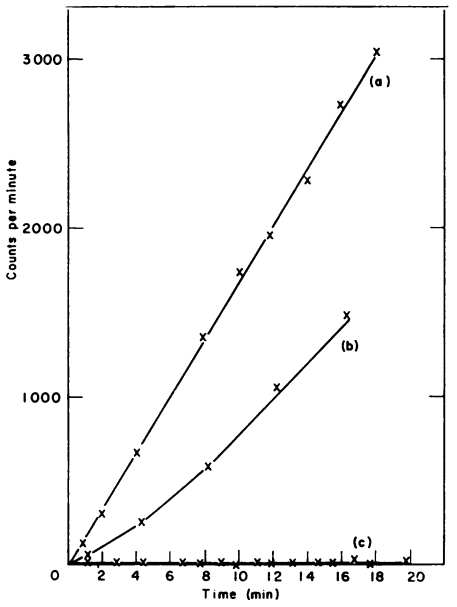


FIG. 1.—C^{14}-uracil at a concentration of 5 μg/ml (specific activity 1.8 μc/μM) was added to exponentially growing *B. subtilis* cultures at time $t = 0$. *Curve a:* the acid-precipitable C^{14} count from 2 ml of culture removed and added to TCA at time t. The count has been normalized to the cell density at the beginning of the experiment. *Curve b:* (See experiment 2 for a complete explanation of this curve) 10 ml of the culture were removed at time t and added to a flask containing 100 μg actinomycin D. Samples were removed from the incubation flask with actinomycin and added to cold TCA 10 and 15 min later. The average of the count obtained on the 2 samples is given as a function of the time at which the sample was added to the antibiotic. *Curve c:* Actinomycin D at a concentration of 10 μg/ml and uracil C^{14} were added to the culture at time $t = 0$.

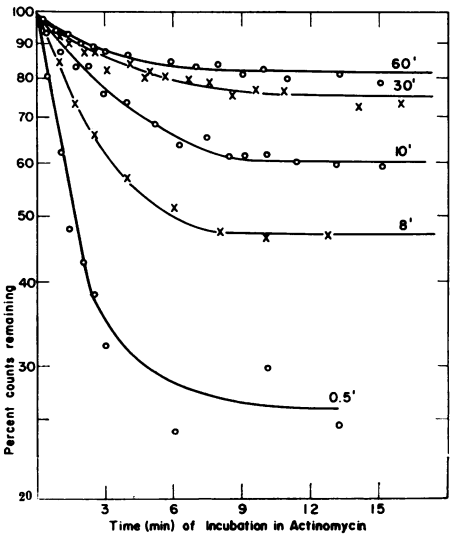
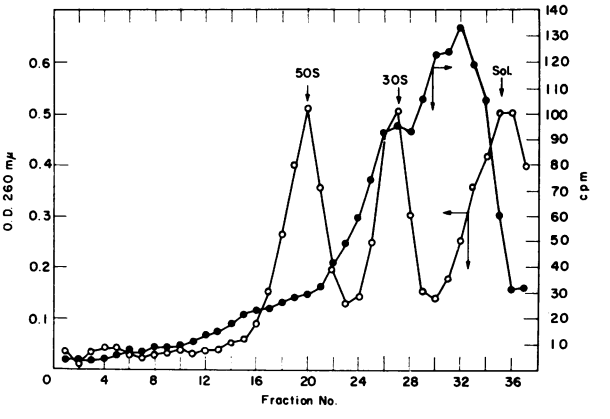


FIG. 2.—C^{14}-uracil was added to cells as in Fig. 1. After various times of preincubation with C^{14}-uracil 40 ml samples were removed and added to a flask with 400 μg actinomycin D. One sample was taken from this flask immediately (time $t = 0$) and others after different times of incubation in actinomycin. The C^{14} count in the acid-precipitable material is given on a logarithmic scale as per cent of the count at $t = 0$. The absolute value of the C^{14} count at $t = 0$ can be estimated from curve *a*, Fig. 1. The preincubation time is given for each curve.

FIG. 3.—Sedimentation analysis of an extract prepared from *B. subtilis* cells labeled for 30 sec with C^{14}-uracil. The extraction and sedimentation in a sucrose gradient was carried out at 2 to 4°C in 10^{-4} *M* Mg, as described under *Methods*. For each fraction 14 drops were collected for counting and 4 drops in 1 ml H_2O for absorption at 2600 Å.



about half of the C^{14} to become incorporated into RNA that is stable on further incubation in actinomycin. A third of the culture was chilled immediately and actinomycin was added to the remainder; this was further divided, one-half being chilled after 1 minute, the other after 10 minutes. Sedimentation analyses of extracts prepared from this experiment are shown in Figures 4(*a*) and (*b*).

It is evident by comparison of Figures 4(*a*), and 4(*b*) that the mRNA does decay

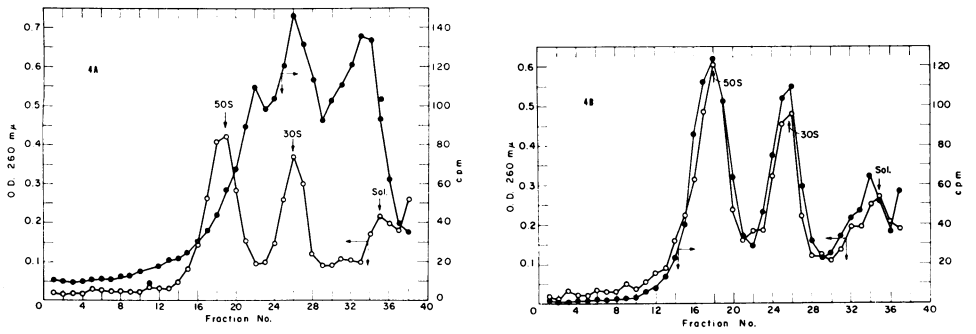


FIG. 4.—Sedimentation analysis of an extract prepared from cells labeled for 3 min with C^{14}-uracil; preparation as in Fig. 3. (*b*): Sedimentation analysis of an extract prepared from cells labeled as in (*a*) and further incubated with actinomycin for 10 min.

The total number of counts recovered per mg of RNA is reduced by a factor of two after the 10-min incubation in actinomycin. The sedimentation pattern and the specific activity obtained after 1-min incubation in actinomycin was intermediate between that of Fig. 4*a* and 4*b*.

in the presence of actinomycin and that after this decay has taken place all the stable RNA fractions are equally labeled. As has been reported for *E. coli*,[18] the 30*S* ribosomes become labeled before the 50*S*. A small amount of label is transferred to the 50*S* material in the presence of actinomycin. When the preincubation is for 30 sec instead of 3 min, a much smaller fraction of the incorporated counts appear in the 30 and 50*S* particles (compare Figs. 3 and 4*a*). Thus, the label transferred into the 50*S* particles does not represent mRNA, but is due to incorporation of labeled ribosomal RNA into the nucleoprotein particles having the sedimentation constant of 50*S*.

Experiment 5: Since the uracil experiments indicated that no new RNA was formed and that pre-existing mRNA decayed in the presence of actinomycin, it was of interest to determine the rate of protein synthesis after addition of the antibiotic. When C^{14}-valine mixed with actinomycin D was added to an exponentially growing culture of *B. subtilis*, the C^{14} incorporation into protein continued for a short time at the same rate as in the control without actinomycin (Fig. 5). The rate of incorporation in the presence of actinomycin decreased rapidly, however, and the total amount of C^{14} amino acid incorporated in 15 min was equal to the amount incorporated by the control in 3–4 min. No further valine incorporation could be detected between 15 and 30 min. In contrast, the addition of chloramphenicol at a concentration of 100 μg/ml instead of actinomycin caused an immediate reduction of the C^{14}-valine uptake rate by a factor of about 50, and incorporation continued at this reduced rate for 20 min.

Analogous experiments carried out with C^{14}-phenylalanine gave similar results.

The amino acid incorporation experiments indicate that in the presence of actinomycin, the rate of amino acid incorporation falls with a decay time of 3–4

min, which is comparable to the 2 min observed for the messenger RNA fraction. The rate of amino acid incorporation is, therefore, approximately proportional to the amount of RNA remaining in the messenger fraction at any time after the addition of actinomycin.

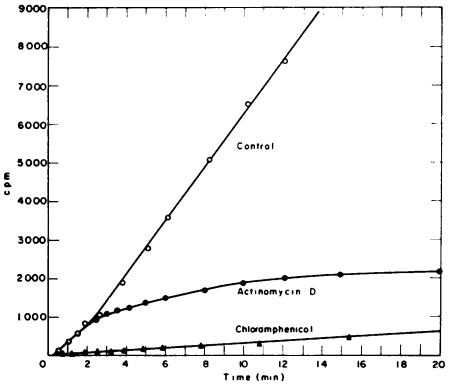


FIG. 5.—Incorporation of C^{14}-valine by exponentially growing *B. subtilis*. At time $t = 0$, 5 μg/ml C^{14}-valine, 0.01 μc/mg, was added. One culture (control) received no antibiotic, a second had actinomycin added at a concentration of 10 μg/ml with the C^{14}-valine, and a third had chloramphenicol at 100 μg/ml added with the valine.

Quantitative Analysis of Results.—Flow of C^{14}-uracil: The uracil incorporation experiments can be summarized as follows. A rapidly labeled RNA fraction decays into acid-soluble material in the presence of actinomycin. This fraction has the same sedimentation properties as those of messenger RNA isolated from *E. coli* using similar methods.[18] In contrast, most of the C^{14}-uracil incorporated into RNA during a prolonged preincubation is stable upon further incubation in the presence of actinomycin, and the stable material has the sedimentation properties of ribosomal and soluble RNA.

The RNA labeling and decay pattern can be understood in terms of the model shown in Figure 6. If we assume that the rate of messenger decay in the presence of actinomycin is the same as that in untreated cells, then the size of the mRNA and stable RNA fractions can be estimated, as well as the various flow rates.

In the steady state the relative sizes of the various pools and flow rates will remain constant, and their absolute values will increase at the same rate as the cell population. If τ is the generation time of the cells (approximately 100 min in these experiments) then each of the pools and each of the flow rates increases as $e^{t/\tau}$.

After a short pulse the mRNA fraction decays exponentially in actinomycin, with a time constant τ_M of about 2 min. The flow rate f_3 must therefore be M/τ_M where M and S refer to the amounts of messenger and stable RNA respectively. The flow f_2 must be greater by the amount required to allow for the growth of the messenger fraction; thus $f_2 = M/\tau_M + M/\tau$ and f_4 the flow into the stable fraction, will be S/τ. Since τ_M is about 2 min and τ is 100 min f_2 is approximately equal to f_3.

The most reliable way of estimating the size of the messenger fraction is by measuring the decay of counts in the presence of actinomycin after sufficiently long preincubation with C^{14}-uracil, so that both the pool and the messenger RNA have the same specific activity as the uracil added to the medium. Under these conditions the ratio of the counts in stable RNA to those of the RNA of the cells should be given by the following expression:[19] cpm stable/cpm total $= 1 - M\, e^{t/\tau}/(M + S)(e^{t/\tau} - 1)$.

According to the results of Figure 2, the observed ratio is 0.82 for $t = 60$ min and 0.75 for $t = 30$ min, giving values of 8 and 9% of the total RNA respectively for the *size* of the messenger fraction.

Flow of uracil into the different RNA fractions: With this value for the messenger fraction, M, we calculate the various flow rates: $f_4 = 0.92\%$ per min, $f_2 = 4.33\%$ per min, and $f_3 = 4.25\%$ per

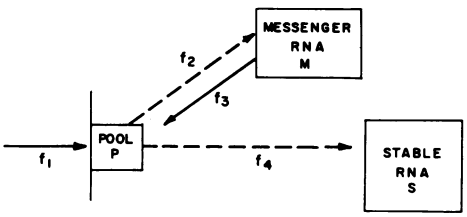


FIG. 6.—A model for the flow of uracil into the various cell fractions. The pool (P) is assumed to contain all of the acid-soluble uracil compounds. f_1 represents the flow of external uracil into the cell. f_2 and f_4 represent the synthesis of high molecular weight RNA from this pool into mRNA and stable (ribosomal and transfer) RNA. f_3 is the breakdown of the mRNA to acid-soluble material. Actinomycin is assumed to stop f_2 and f_4 and to leave the initial rate of f_3 unchanged.

min, where all flow rates are given as percentages of total RNA per min. Thus, with a very short pulse, about 80% of the C^{14} in the high molecular weight RNA should be in messenger. This is in approximate agreement with the data of Figure 2 and consistent with the sedimentation analysis of the extract from the 30-second pulse (Fig. 3).

Number of times a messenger molecule functions: The incorporation of C^{14}-valine into cell protein can be taken as a measure of the rate of protein synthesis, except for the fact that there is a pool of internal valine which produces an initial lag in the C^{14}-valine incorporation (see Fig. 5). There is some indication that a slow incorporation of C^{14}-valine continues in the presence of actinomycin D beyond the point where all of the mRNA has decayed. However, neither the experiments with labeled amino acid nor those with labeled uracil are sufficiently precise to determine whether there exists a small fraction of more stable messenger or a small amount of protein synthesis which is not dependent on unstable messenger. An answer to this question will require a study of the nature of the proteins formed at various stages after the addition of actinomycin D.

The unambiguous conclusions from the amino acid experiments are the following: Immediately after the simultaneous addition of C^{14} amino acid and actinomycin, incorporation takes place at a rate identical with that in the control. The total incorporation stops within a short time, when the amount of C^{14} amino acid incorporated into protein is about the same as that incorporated in 4 minutes in the absence of actinomycin.

If we assume that the formation of new messenger is totally stopped by the antibiotic, then the residual protein synthesis after addition of actinomycin must be due to the functioning of the messenger which existed in the cells at the time of the actinomycin addition. About 4% of the total cell protein is made by this pre-existing messenger; hence, we can estimate the average number of times a messenger molecule must function. For example, a culture containing 100 grams of protein makes 4 grams of new protein using 2.0 grams of pre-existing messenger (8% of 25 grams).[20] If each mRNA molecule were able to function only once in the formation of protein, then 36 grams of mRNA would have been required for the 4 grams of residual protein synthesis after actinomycin has been added to the culture. This calculation assumes that each amino acid is coded by 3 nucleotides, taking the mean nucleotide molecular weight as 3 times the mean molecular amino acid weight, so that the RNA must be 9 times heavier than the protein it determines. Thus, we conclude that *on the average* a messenger molecule can function approximately 10–20 times. It does not seem likely that this average value could be due exclusively to a small fraction of the protein being produced by a totally stable RNA template since the total rate of synthesis during the first minute after adding C^{14}-valine is the same with and without the simultaneous addition of actinomycin.

The time required to form protein molecules in growing *E. coli* has been estimated[21] as 5–10 sec. A mean decay time of approximately 2 min for messenger is thus consistent with the hypothesis that each messenger molecule makes approximately 10–20 protein molecules before it is broken down to acid-soluble material.

Discussion.—Since RNA viruses can multiply in the presence of actinomycin and since DNA-dependent RNA synthesis is totally suppressed by actinomycin in cell-free systems, it is reasonable to interpret the RNA inhibition in *B. subtilis* as due to an interference with a DNA-dependent process. The fact that all RNA synthesis

is stopped would then imply that ribosomal and transfer RNA are made by copying directly from DNA rather than being dependent upon a system that copies RNA from RNA.

The breakdown of mRNA to small molecules has been inferred from the shifts in the proportions of P^{32}-labeled nucleotides in RNA, when the isotope incorporated during a pulse is chased into stable RNA upon further incubation in a medium with nonradioactive phosphorus.[6, 18] The results obtained here indicate that the breakdown of mRNA to acid-soluble material does take place. It appears that the mRNA exhibits the kinetic behavior of a direct precursor[10] of the stable material only because it becomes equilibrated with the pool of low molecular weight RNA precursors. The time constant for this breakdown, which can be measured in the presence of actinomycin, is consistent with the kinetics of the chase experiments but cannot be determined from them since a given uracil molecule is generally used several times in mRNA before it becomes incorporated into stable material.

It must be stressed that this interpretation of the nature of the decay of mRNA is based on the assumption that the phenomena observed in the presence of actinomycin are representative of the reactions which occur in untreated cells. More specifically, it is based on the assumption that only the DNA-dependent RNA synthesis is blocked by this antibiotic. The validity of this assumption, however, is not critical for the determination of the number of times the messenger molecules function, since these calculations are based only on the fact that no RNA is synthesized after the addition of actinomycin.

Since a messenger molecule appears to function on the average of 10 to 20 times in protein synthesis, one may ask whether the cell has some mechanism for counting, say, to 15, or whether the breakdown of mRNA is a random process with a mean life of approximately 2 min. The fact that after a 30-sec pulse the exponential decay starts immediately suggests that the decay does not wait until a prescribed number of protein molecules have been made, but that it probably occurs at random. Although the mechanisms of the breakdown process remain unknown, the fact that one does not observe in the sucrose gradients any large accumulation of low molecular weight, acid-precipitable material when a part of the mRNA labeled during a short pulse has become acid-soluble, indicates that each mRNA molecule is hydrolyzed rapidly once the process is initiated. This could be due either to the nature of the degradative enzyme or to a protection of the mRNA as long as it remains attached to a ribosome.

In some mammalian cells, particularly those which are concerned with the manufacture of a few types of protein, there is no evidence of an RNA fraction which turns over rapidly. For example, rapidly labeled messenger-like RNA has not been found in reticulocytes[22, 23] or rat liver cell cytoplasm[24] while it has been observed in the nuclear RNA of liver cells[24] and in HeLa cells grown in tissue culture.[25] In view of the results reported here it is of particular interest that protein synthesis in reticulocytes is not inhibited by actinomycin,[22] a finding which supports the hypothesis of a stable RNA template.

Since bacteria respond to changes in external environment by altering their protein composition, the advantage of a mechanism for degrading the template RNA is clear. If, however, there is no necessary connection between breakdown of the template and protein synthesis, the breakdown need only be sufficiently rapid to

allow adequate response to changes in the environment, and no system has been described in which the response time is less than several minutes.[26] Therefore, the mean life of 2 minutes observed for mRNA in these experiments is consistent with the hypothesis[1] that induction and repression of specific enzymes in bacteria are effected through alterations of the rate at which the various genes function in the formation of messenger RNA.

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[19] This equation can be derived in a method similar to that used in ref. 10 and can be understood by the following argument: Since M and S refer to the amounts of mRNA and stable RNA at $t = 0$ when the label is added, the amount of mRNA at a later time is $Me^{t/\tau}$ and the C^{14} contained in it is $\mu Me^{t/\tau}$ where μ is the specific activity of the uracil added. For the stable fraction the incorporated counts will equal the specific activity of uracil multiplied by the amount of newly synthesized RNA minus an amount of C^{14} needed to fill the pre-existing pool of mRNA or: *cpm stable* $= \mu S\,(e^{t/\tau} - 1) - \mu M$.

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