Lifetime of Bacterial Messenger Ribonucleic Acid

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ABSTRACT

Moses, V. (University of California, Berkeley), and M. Calvin. Lifetime of bacterial messenger ribonucleic acid. J. Bacteriol. 90:1205–1217. 1965.—When cells from a stationary culture of Escherichia coli were placed in fresh medium containing inducer for β-galactosidase, growth, as represented by increase in turbidity and by total protein synthesis, started within 30 sec. By contrast, β-galactosidase synthesis was greatly delayed compared with induction during exponential growth. Two other inducible enzymes (D-serine deaminase and L-tryptophanase) and one repressible enzyme (alkaline phosphatase) showed similar lags. The lags were not due to catabolite repression. They could not be reduced by pretreatment of the culture with inducer, or by supplementing the fresh medium with amino acids or nucleotides. The lag was also demonstrated by an I^- mutant constitutive for β-galactosidase synthesis. An inhibitor of ribonucleic acid (RNA) synthesis, 6-azauracil, preferentially inhibited β-galactosidase synthesis compared with growth in both inducible and constitutive strains. Puromycin, an inhibitor of protein synthesis, acted as an inhibitor at additional sites during the induction of β-galactosidase synthesis. No inhibition of the reactions proceeding during the first 20 sec of induction was observed, but puromycin seemed to prevent the accumulation of messenger RNA during the period between 20 sec and the first appearance of enzyme activity after 3 min. It is suggested that these observations, together with many reports in the literature that inducible enzyme synthesis is more sensitive than total growth to some inhibitors and adverse growth conditions, can be explained by supposing that messenger RNA for normally inducible enzymes is biologically more labile than that for some normally constitutive proteins. The possible implications of this hypothesis for the achievement of cell differentiation by genetic regulation of enzyme synthesis are briefly discussed.

During the past 2 or 3 years, reports from a number of laboratories have shown that the synthesis in bacteria of inducible enzymes is generally more sensitive to the presence of certain inhibitors than is growth as a whole (Engelberg and Artman, 1964; Henderson, 1962; Paigen, 1963; Pardee and Prestidge, 1963; Sypherd and DeMoss, 1963; Sypherd and Strauss, 1963a, 1963b; Sypherd, Strauss, and Treffers, 1962). Paigen (1963) found that inducible enzyme synthesis was inhibited by leucine, valine, histidine, and serine, and was promoted by iodoacetate. This was interpreted in terms of a catabolite repression effect (Magasanik, 1963). Pardee and Prestidge (1963) observed that, compared with growth, β-galactosidase synthesis in Escherichia coli is preferentially inhibited by ultraviolet irradiation, and they also invoked an explanation based on catabolite repression. The other workers mentioned above, employing as inhibitors deuterium oxide, streptomycin, chloramphenicol, puromycin, tetracycline, etc., have more or less explicitly rejected catabolite repression. Indeed, Engelberg and Artman (1964), working with streptomycin, proposed an alternative explanation based on the concept of varying biological stabilities or lifetimes for different specific messenger ribonucleic acid (mRNA) molecules.

The concept of long-lived mRNA is common in the consideration of higher organisms. For example, Spencer and Harris (1964) showed that protein synthesis proceeds in cells of the giant alga Acetabularia crenulata for days after enucleation, and Prescott (1959, 1960), and Goldstein, Micou, and Crocker (1960), found protein synthesis to continue for many hours after the removal of the nuclei from amoebas and from human amnion cells. One could always argue, however, that, in view of the far more rapid growth rate of bacteria, a short-lived bacterial mRNA, with a life of minutes, is functionally equivalent to a mRNA species in a higher organism having a life-time of hours or days. It therefore became of particular interest to investigate the possibility of a range of stabilities among different mRNA functions within one organism.
Materials and Methods

Organisms and growth conditions. The following strains of E. coli were used: C600-1 (i'(y-z-B)\textsuperscript{r}), from A. B. Pardee; ML-3 (i'y-z\textsuperscript{r}), and from J. A. Clark; Cavalli (i'y-z'met-thy\textsuperscript{r}), from Aileen Simmons; C90F1 (i'zP'R\textsuperscript{r}), from A. Garen. All except ML-3 and C90F1 were grown on M63 medium, containing ammonium sulfate, mineral salts, and glycerol, with thiamine, methionine, and thymine added as necessary (Pardee and Prestidge, 1961). Strain ML-3 was grown in the maltose-salts medium described by Boezi and Cowie (1961). Strain C90F1 was grown on TGE medium of Garen and Siddiqi (1962), with 0.1% glycerol instead of glucose, a phosphate concentration of 5 \times 10\textsuperscript{-4} M, and omission of the amino acids. Growth was at 37 C in air with constant stirring, and with formation of lumps when measuring optical density in 1-cm cuvette at 650 m\textmu. An optical density of 1.0 was equivalent to a bacterial concentration of about 0.43 mg (dry weight) per ml.

Enzyme induction. The kinetics of \(\beta\)-galactosidase induction were obtained by use of isopropylthio-\(\beta\)-D-galactopyranoside (IPTG), usually at 5 \times 10\textsuperscript{-4} M, or methylthio-\(\beta\)-D-galactopyranoside (TMG), 10\textsuperscript{-2} M, as inducers. After addition of the inducers, 0.2-ml samples of the suspension were transferred to tubes containing 20 \textmu l of tolune together with 20 \textmu l of an aqueous solution of cysteine (0.05 M) and Triton-X100 (0.5%). The contents of the tubes were violently agitated for 30 sec with a Vortex mixer.

For measurement of enzyme activity, 0.8 ml of the following solution was added to each tube: 0.0167 M K\textsubscript{2}HPO\textsubscript{4}, 0.0833 M K\textsubscript{2}HPO\textsubscript{4}, 0.125 M NaCl, and 0.0033 M o-nitrophenyl-\(\beta\)-D-galactopyranoside. The tubes were incubated with shaking at 37 C until sufficient yellow color had developed, and the reaction was stopped by adding 0.2 ml of 1.5 M Na\textsubscript{2}CO\textsubscript{3}. The reaction time for each tube was noted. The tubes were centrifuged at 10,000 \times g for 15 min, and the absorbance of the clear supernatant solution was determined at 420 m\textmu.

Tryptophanase induction and assay, with L-tryptophan (300 \mu g/ml) as inducer, were performed with strain C600-1 as described by Pardee and Prestidge (1961). p-Serine deaminase was induced with d-serine (300 \mu g/ml) in strain ML-3 and was assayed as described by the same authors (Pardee and Prestidge, 1955). Alkaline phosphatase was measured by the hydrolysis of p-nitrophenyl phosphate in tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8 (Garen and Siddiqi, 1962). The reaction time was 15 min. The reaction was stopped by adding sufficient NaOH to raise the pH to about 14, and after centrifugation the absorbance of the supernatant fluid was determined at 420 m\textmu. Units of enzyme activity are expressed in all cases as millimicro moles of substrate metabolized per minute per milliliter of cell suspension at 37 C.

Chemical determinations. For measurement of protein and nucleic acid, 5-ml samples of bacterial suspension were mixed with cold trichloroacetic acid to give a final concentration of about 5%. These samples were later analyzed for protein, RNA, and deoxyribonucleic acid (DNA) as described by Berrah and Konetzka (1962).

Radioactivity experiments. For measurements of the incorporation of L-methionine-C\textsuperscript{14}H\textsubscript{4}, the medium contained 0.4 \mu mole (2.5 \mu c) of methionine per ml. For uracil-\(\beta\)-C\textsuperscript{14} incorporation, the concentration of the pyrimidine was 0.2 \mu mole (0.5 \mu c) per ml. At intervals, 0.25-ml samples of cell suspension were mixed with 0.25 ml of cold 10\% (w/v) trichloroacetic acid. After remaining at 0 C for about 30 min, the sample was filtered through a prewetted Millipore filter (0.45-\mu m size, 25-mm diameter). The cells were transferred onto the filter with two washes of cold 5\% trichloroacetic acid, each of 1 ml. After removal of the acid by suction, the sample on the filter was washed with 3 ml of water, followed by seven 1-ml washes of water. The filter was sucked dry for 1 min and then the dried residue was dissolved in 18 ml of scintillation fluid no. 2 with vigorous shaking. The contents of the vial were solidified with thixotropic gel powder (Cab-O-Sil; Packard Instrument Co., La Grange, Ill.), maintaining the bacterial cells in suspension. Radioactivity was counted in a Packard scintillation counter with an external standard. Analysis of the scintillation samples, of the precipitated cells, demonstrated that both methionine and uracil were incorporated essentially unchanged into macromolecules.

Cell counts. Samples for determination of cell number and volume were taken into growth medium containing sufficient formaldehyde to give a concentration of 0.2% after mixing with 0.4% Na\textsubscript{2}CO\textsubscript{3} (Lark and Lark, 1960). The reaction was stopped by adding 0.25 ml of cold 10\% trichloroacetic acid. The mixture was filtered through a 0.4-\mu m filter (Millipore Filters) and the cell number counted in a Coulter Electronic Counter System I (Radiation Instrument Development Laboratory, Melrose Park, Ill., no. 018039), a Four Hundred Channel Pulse Height Analyzer (RIDL no. 34-12B), and a Digital Recorder (Hewlett-Packard, Palo Alto, Calif., no. H43-502A). The particle sizes were experimentally distributed into 100 channels, and two parameters were measured after subtraction of background noise: the sum of the particles in all channels, and the sum of the products of each channel number and the number of particles in that channel. Since each channel number is directly proportional to the volume of the individual particles giving rise to pulses falling in that channel, the product of the channel number and the number of particles gives an arbitrary measure of the total bulk of cell material in that particular channel. Summation of these values for all channels then gives the total bulk of bacterial substance in the suspension in arbitrary units.
An average cell volume may also be calculated for each sample.

RESULTS

β-Galactosidase induction and its relation to growth. In the media used in the present work, E. coli grew exponentially as measured by increase of absorbance, with a doubling time at 37 C of 65 to 85 min. Growth eventually stopped as a result of the exhaustion of the glycerol in the medium; the nitrogen source was present in great excess. When growth stopped, the optical density remained approximately constant for a prolonged period. If, after remaining for about 1 hr in the stationary phase of growth, a portion of the culture was rapidly added to several volumes of fresh growth medium, an exponential increase in absorbance of the culture began immediately, the first measurement usually being about 30 sec after the addition to allow for thorough mixing (Fig. 1). Under these conditions of starvation, therefore, there was no lag before growth resumed when stationary cells were reintroduced to a medium favorable for growth.

The introduction of inducer to an exponentially growing culture typically initiates the sudden onset of enzyme protein synthesis which starts 2.5 to 3 min later (Boezi and Cowie, 1961; Nakada and Magasanik, 1964; Pardee and Prestidge, 1961). We also found this to be true (e.g., Fig. 2), whether a small volume of a concentrated inducer solution was added to the cells, or the cells were diluted many times into fresh medium containing inducer. Monod, Pappenheimer, and Cohen-Bazire (1952) used the differential rate of enzyme synthesis (the rate versus the rate of growth, determined in our case by increase in optical density) as a means of comparing the synthesis of a specific protein with the aggregate synthesis of many proteins contributing to growth. In the case of β-galactosidase induced during logarithmic growth, the time required for the differential rate of synthesis to increase from a low basal rate to a steady-state high rate of induced synthesis was very short indeed, probably not more than a few seconds (Fig. 2). A different pattern of differential enzyme synthesis kinetics was observed when cells from a stationary-phase culture were added to fresh medium containing inducer for

![Fig. 1. Growth of Escherichia coli C600-1. Optical density at 660 mμ measured during growth at 37 C. In Fig. 1A, a portion of the stationary culture was diluted fivefold at about 4 hr into fresh medium. In Fig. 1B, glycerol (curve A) or ammonium sulfate (curve B) was added to a stationary culture as indicated by the arrow.](http://jb.asm.org/)

![Fig. 2. β-Galactosidase induction in Escherichia coli C600-1. Inducer (TMG) added at arrow to exponentially growing culture. Differential synthesis of enzyme plotted; mass doubling time was 78 min. The increase in rate of enzyme synthesis took place between 2.5 and 3 min after inducer was added.](http://jb.asm.org/)
$\beta$-galactosidase. Exponential growth started immediately (Fig. 1) and, although $\beta$-galactosidase activity began to appear after 3 min, the differential rate of induced enzyme synthesis gradually increased and did not become constant until about 10 to 25 min after induction started (Fig. 3). This was true for all six strains of E. coli which were tested.

Addition of inducer to cells in the stationary-growth phase permitted only extremely slow synthesis of enzyme (Fig. 4). Nevertheless, contact with the inducer during the stationary phase did not reduce the lag preceding the attainment of a constant differential rate of enzyme synthesis when growth was resumed by dilution into fresh medium containing inducer. Even the continued presence of inducer, originally added during the previous growth phase before the exhaustion of carbohydrate took place, had no effect on the lag when growth was resumed in the presence of inducer (Fig. 5). This was demonstrated by adding IPTG to the culture during exponential growth. The synthesis of $\beta$-galactosidase commenced in 3 min, and the differential rate of synthesis remained constant throughout the rest of the growth period (about 2.5 hr). Eventually, growth stopped and $\beta$-galactosidase synthesis ceased.

After about 1 hr of starvation, part of the culture was diluted into fresh medium still containing IPTG. Growth resumed immediately, but the differential rate of $\beta$-galactosidase synthesis behaved as described earlier, and did not become constant until about 18 min after growth resumed. In the control in this experiment, in which no IPTG was present before dilution, the lag was 14 min (Fig. 5).

Behavior of other enzymes. The lag in enzyme synthesis after resumption of growth was not restricted to $\beta$-galactosidase. Precisely the same kinetic pattern was found with two other inducible enzymes, $\beta$-serine deaminase (in strain ML-3) and L-tryptophanase (in strain C600-1); these showed lags of 19 and 33 min, respectively. When induced in exponentially growing cultures, both of these enzymes demonstrated the typical 3-min response to the addition of the appropriate inducer.

Alkaline phosphatase, in a constitutive strain (C90F1), showed a lag of about 30 min, compared with 21 min for $\beta$-galactosidase in the same culture (Fig. 6).

Effect of the medium. It was necessary to consider the possibility that the lag with $\beta$-galactosidase was in some way the consequence of trans-
ferring the cells from exhausted medium to fresh medium. Exhausted medium was shown by Freter and Ozawa (1963) to be capable of supporting further bacterial growth after removal of the original population by filtration or centrifugation. They suggested that this was the result of aeration of the medium during manipulation.

Stationary cells were therefore added to the filtered exhausted medium containing inducer. A short period (15 min) of very rapid growth did ensue, after which the growth rate fell very rapidly (Fig. 7). The differential rate of β-galactosidase synthesis, however, showed the usual pattern with a lag of 16 min, compared with 14 min for the control (Fig. 8).

Effect on the lag of growth supplements. The presence in the fresh medium of either an enzymatic hydrolysate of casein (200 μg/ml), or of a mixture of the diphosphates of adenosine, cytidine, guanosine, and uridine (128 μg/ml of each), had no effect on the lag or on growth. Although amino acids in the medium are known to be concentrated within the cells (Britten and McClure, 1962), it is very likely that nucleoside diphosphates do not rapidly penetrate the cell membrane, so that their failure to influence the lag cannot be unequivocally assessed. Attempts to overcome penetration difficulties and to provide RNA precursors by supplying a mixture of the four ribonucleosides (50 μg/ml of each) were not successful. These compounds are not normal metabolic intermediates, and, although not af-

Effects of 6-azauracil on enzyme induction and
growth. *E. coli* is not normally affected by actinomycin D (Hurwitz et al., 1962), and a study was made of the effects on the lag of another inhibitor of RNA synthesis, 6-azauracil. This compound is reported to inhibit pyrimidine biosynthesis by being converted to 6-azauridine-5'-phosphate and blocking orotidylic acid decarboxylase (Habermann, 1961). When added to a stationary culture at a concentration of 25 μg/ml, 30 min before dilution into fresh medium containing inducer, 6-azauracil did not immediately affect either the kinetics of growth or of β-galactosidase synthesis. About 44 min after dilution, however, the growth rate suddenly fell by 65% (Fig. 9). The differential rate of β-galactosidase synthesis was identical with that of the control for the first 27 min, and then suddenly fell by 90% (Fig. 10). Since 6-azauracil took so long to act, it could not be used to study RNA synthesis immediately after induction. When eventually it did become inhibitory, it exerted its inhibition sooner and more powerfully on induced enzyme synthesis than on growth, as demonstrated by the 90% fall in the rate of differential enzyme synthesis. If 6-azauracil was added to induced exponentially growing cells, growth was inhibited after about 30 min and β-galactosidase synthesis a few minutes earlier. When 6-azauracil was added to the culture, it seemed that 30 min of active metabolism must occur before inhibition of growth or enzyme synthesis was observed. Thus, when it was added to a growing population inhibition started in 30 min, but when it was introduced into a stationary culture inhibition started only

**FIG. 8.** β-Galactosidase induction on transfer to exhausted medium. Same experiment as shown in Fig. 7; in both cases IPTG added at time of dilution. Differential synthesis of enzyme plotted intermittently. Curve A, dilution into fresh medium at arrow A; differential rate of enzyme synthesis became constant after 15 min. Curve B, dilution into exhausted medium at arrow B; differential rate of enzyme synthesis became constant after 15 min.

**FIG. 9.** Effect of 6-azauracil on resumption of growth. Stationary culture of *Escherichia coli* C600-1 diluted into fresh medium. A, control; B, 6-azauracil (25 μg/ml) was added to the stationary culture 80 min before dilution (as indicated by arrow), and was also present at the same concentration in the fresh medium.

**FIG. 10.** Effect of 6-azauracil on β-galactosidase synthesis during resumption of growth. Same experiment as Fig. 9. In both cases IPTG was added at time of dilution. A, control; B, plus 6-azauracil. Differential synthesis of enzyme plotted at 3-min intervals.
after 30 min of subsequent growth had occurred (Fig. 9 and 10).

Behavior of an $i^-$ constitutive strain. When a strain constitutive for $\beta$-galactosidase was diluted into fresh medium after remaining stationary for 1 hr, there was an immediate resumption of exponential growth, but there was a lag of 42 min before the differential rate of $\beta$-galactosidase synthesis became constant (Fig. 11). The inhibitory effect of 6-azauracil was similar to that in the inducible strains; after 30 min, growth was partially inhibited and the differential rate of enzyme synthesis dropped to zero.

Growth and macromolecular syntheses during growth transitions. Studies were made of a number of constitutive growth characteristics during the rapid transition from a stationary culture to an exponentially growing one. Figure 12 compares the responses of optical density of the culture, concentration of cells, and total cell bulk. Changes in neither the number of cells nor in the total cell volume corresponded with the immediate exponential increase in optical density. Both of

![Figure 11. $\beta$-Galactosidase synthesis during resumption of growth of a constitutive strain, and effect of 6-azauracil. Escherichia coli 290U ($i^-$ constitutive) diluted from stationary phase into fresh medium. A, control; B, plus 6-azauracil (25 $\mu$g/ml) originally added 30 min before dilution and also present in the fresh medium. Differential synthesis of enzyme plotted at 3-min intervals for curve A. For curve B some points omitted owing to lack of space; $\beta$-galactosidase synthesis occurred only from about 23 to 31 min after dilution.](http://jb.asm.org/)

![Figure 12. Population kinetics and macromolecular syntheses after resumption of growth. Stationary cultures of Escherichia coli C600-1 were diluted into fresh medium. In experiment A, measurements were made at intervals of the optical density of the suspension, cell concentration, total cell volume, and average cell volume; in experiment B, determinations were made of protein, RNA, and DNA.](http://jb.asm.org/)

these parameters exhibited long delays (34 and 19 min, respectively) before they began to increase logarithmically. An investigation was also made of the levels of total DNA, RNA, and protein in the culture after the growth transition. Kinetic measurements of constitutive parameters usually exhibited considerable scatter because of the relatively small increases between successive samples. As we wished to collect samples at 2-min intervals, all volumetric measurements in this experiment were confirmed gravimetrically. The results (Fig. 12) demonstrate that there was no lag in the initiation of protein synthesis. A similar result was obtained by Hershey (1938). DNA exhibited a lag of 27 min, and there may have been a short lag of about 5.5 min for RNA.

The absence of a lag in protein synthesis was confirmed by measurement of the incorporation of methionine-$^{14}CH_3$ (Fig. 13). The incorporation of uracil-$^{14}CH_4$ showed an initial low differential rate of RNA synthesis, which finally became constant and maximal 28 min after resumption of growth (Fig. 13).

Effects of puromycin on growth and on inducible
enzyme synthesis. The induced synthesis of $\beta$-galactosidase in *E. coli* is more sensitive than growth to the presence of puromycin (Table 1). When puromycin ($5 \times 10^{-4}\text{M}$) was added to a logarithmically growing culture of cells which were also inducibly synthesizing $\beta$-galactosidase, enzyme synthesis terminated instantly, and growth as measured by turbidity slowed down but did not stop entirely for over 1 hr (Fig. 14).

Kepes (1963) showed that, when a culture inducibly synthesizing $\beta$-galactosidase is suddenly diluted 50-fold to reduce the inducer concentration to a level too low to promote induction, the rate of enzyme synthesis begins to slow down immediately and comes to a complete halt in a few minutes. When such dilution to reduce the inducer concentration was performed after only 2 to 3 min of contact between the cells and the inducer, and before enzymatic activity had appeared, then a short burst of enzyme synthesis was observed. This began about 3 min after the original introduction of inducer to the culture and ceased a few minutes after dilution had taken place. The burst of enzyme synthesis is interpreted as being the translation into protein of mRNA formed during the time of contact with inducer. This translation did not commence until

### Table 1. Effect of puromycin on the growth and inducible synthesis of $\beta$-galactosidase in *Escherichia coli* C600-1

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<th>Purmcytin conc</th>
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* Growth was measured by turbidity at 650 mg. Differential rate of $\beta$-galactosidase synthesis expressed as rate of synthesis of enzyme activity per unit increase of turbidity.
3 min after the introduction of inducer. Net mRNA decay started as soon as inducer was withdrawn, and none was left within a few minutes. During this period, the rate of enzyme synthesis fell in proportion to the decreasing concentration of mRNA, and when the mRNA was all gone enzyme protein synthesis ceased entirely.

If $5 \times 10^{-4}$ M puromycin and inducer were both in contact with the cells for 3 min, no burst of enzyme synthesis was observed after simultaneous dilution of both puromycin and inducer, even though growth did resume immediately after dilution of the antibiotic (Fig. 15). The low concentration of puromycin after dilution ($10^{-9}$ M) was not inhibitory. In this experiment, inducer was added to the cells 1 min after puromycin, and dilution took place 3 min later; the total contact time between puromycin and the cells was thus 4 min. In another experiment, $5 \times 10^{-4}$ M puromycin was incubated with cells for 4 min, and the suspension was then diluted 50 times into medium containing $5 \times 10^{-4}$ M IPTG but no puromycin. Growth resumed immediately, and there was no perceptible delay in the onset of enzyme synthesis compared with a control sample. Thus, 4 min of contact with puromycin is not sufficient to produce a differential discrimination against the induction and synthesis of $\beta$-galactosidase immediately after removal of the antibiotic. A longer contact time of 32.5 min before removal of the puromycin and addition of inducer did result in a delay of about 26 min before enzyme synthesis started.

If puromycin was added 2.5 min after inducer, just before the time that enzyme protein synthesis was beginning, and both puromycin and inducer were removed by dilution at 3 min, a slight diminution in the subsequent burst of enzyme synthesis was observed (Fig. 16), indicating that some degree of inhibition may take place in 30 sec of contact time. However, when inducer was added to the culture 30 sec after puromycin, and both were removed by dilution 20 sec later, there
was no observable inhibition of the subsequent burst of enzyme synthesis (Fig. 17).

**DISCUSSION**

*Enzyme synthesis after resumption of growth.*

The lag in the attainment of a constant differential rate of synthesis shown by all inducible and repressible enzymes examined appears not to be the direct result of an environmental change for the cells when they are transferred from a medium deficient in glycerol to one containing sufficient carbohydrate for growth. The observation that an exponentially growing culture shows no prolonged lag when diluted many times into fresh medium containing inducer, whereas a culture from a glycerol-deficient medium still shows a lag when diluted into exhausted medium with inducer, indicates that medium shifts per se are not responsible for the lag.

A second possibility to be considered is that catabolite repression (Magasanik, 1963) develops during the stationary phase and that on resumption of growth the catabolite repressor must be removed by metabolism before \( \beta \)-galactosidase induction can occur. Such an induction lag, resulting from catabolite repression, was demonstrated by Nakada and Magasanik (1964) in a threonine-requiring mutant of *E. coli* when starved of threonine in the continued presence of glycerol (the energy and carbon source). No catabolite repression was observed under any circumstances when the cells were starved of glycerol. Pardoe and Prestidge (1963) and Mandelstam (1961) also showed that catabolite repression does not occur in the absence of a carbon source. In the current work, in which the cells were shown to stop growing because of exhaustion of the carbon source (Fig. 1), we may thus exclude the possibility of the development of catabolite repression during the starvation period.

Further support for this conclusion comes from the studies of Attardi et al. (1963). They showed that when *E. coli* was grown in a medium containing glucose and lactose, a diauxic growth curve resulted as a consequence of the catabolite repression of \( \beta \)-galactosidase synthesis, so long as glucose remained in the medium. When all the glucose had been used and growth had ceased, then a very rapid preferential induced synthesis of \( \beta \)-galactosidase took place. We may conclude that catabolite repression is very rapidly relieved when the usable carbon source is exhausted. Finally, on this point, we have shown directly that alkaline phosphatase, whose synthesis is not affected by catabolite repression (McFall and Magasanik, 1960), demonstrates a lag in the same way as does \( \beta \)-galactosidase (Fig. 6).

In searching, then, for an explanation of the prolonged lag which would fit all the observed facts, we have considered the possibility that carbohydrate starvation, depriving the cells of both carbon and energy, results in a depletion of pools of intermediary metabolic precursors required in the synthesis of macromolecules. Such pools would be replenished on the restoration of carbohydrate, and the increasing rate of \( \beta \)-galactosidase synthesis might reflect the increasing size of one or more of these pools. Obvious candidates for such precursors are amino acids for protein synthesis and ribonucleotides for RNA synthesis, since both of these types of macromolecules are made when enzymes are induced.

As far as the pools of amino acids are concerned, there seems no reason to suppose that in our system these even limit the rate of protein synthesis. Amino acids in the medium are readily concentrated within the cells by *E. coli* (Britten and McClure, 1962), yet the lag was not curtailed by the presence of amino acids in the medium. Total protein synthesis showed no lags (Fig. 12). The incorporation of \(^{14} \text{C}\)-methionine into protein also demonstrated no lag (Fig. 13). These observations all indicate that no shortage of amino acids existed as the cells were transferred into fresh medium.

There is more reason to believe that ribonucleotide pools might be seriously depleted during starvation and require replenishment when growth starts. Total RNA is known to fall during starvation (Dawes and Ribbons, 1965). All those proteins dependent on labile mRNA, and which require a continuous supply of mRNA for a constant rate of production, showed a lag. This applies equally to inducible enzymes (\( \beta \)-galactosidase, \( \delta \)-serine deaminase, and \( \lambda \)-tryptophanase) and to repressible enzymes (alkaline phosphatase). Some lag is also shown in the rate of total RNA synthesis (Fig. 12) and in the incorporation of labeled uracil (Fig. 13). During starvation, mRNA does not accumulate in the presence of inducer, or in a constitutive strain, even though catabolite repression is absent. If messenger did accumulate, one would expect an immediate synthesis of the preinduced enzyme on restoration of the carbohydrate source. Such an immediate synthesis does occur with some proteins (e.g., total protein), but not with those known to be under inducible or repressible control.

The failure of ribonucleoside diphosphates in the medium to shorten the lag is probably due to their lack of penetration into the cells. Unpublished experiments in this laboratory have shown that the metabolism by *E. coli* of phosphorylated intermediates added to the medium is very slow,
even though the same substances are rapidly metabolized when formed inside the cell.

We may then tentatively conclude that the lag originates in the failure of the cells to synthesize mRNA at the normal rate as a consequence of the depletion of their ribonucleotide pools. Since the syntheses of some proteins are not subject to lags, we must presume that these proteins are not dependent on ribonucleotide pools because their mRNA is already present. We have already remarked that mRNA appears not to be made during starvation, and we therefore infer that those mRNA species existing at the end of a period of carbohydrate starvation were made during an earlier growth period, and must thus be considered biologically stable, at least as compared with \(\beta\)-galactosidase messenger.

If this explanation is correct, one would not expect the lag to be affected by mutation of the regulator gene controlling the inducible enzyme synthesis. This gene is believed to control only the initiation of DNA-dependent mRNA transcription. As predicted, an \(i^s\) constitutive strain of \(E.\ coli\) showed the same lag for \(\beta\)-galactosidase synthesis (Fig. 11) as did the corresponding inducible strain (Fig. 3). Further, it should be possible to inhibit inducible enzyme synthesis preferentially, and thus prolong the lag, by the action of an inhibitor of mRNA synthesis. 6-Azauracil, an inhibitor of pyrimidine biosynthesis, and hence of RNA synthesis, did indeed preferentially inhibit \(\beta\)-galactosidase synthesis compared with growth. Since 6-azauracil did not exert its inhibitory effect until after 30 min of active metabolism, it could not be tested during the critical first few minutes after growth resumed.

Many differential effects have already been noted between induced enzyme synthesis and growth which might be ascribed to such variation in mRNA stabilities. Actinomycin D has been shown to inhibit induced histidine synthase more than growth in \(Bacillus subtilis\) (Hartwell and Magasanik, 1963), and also to be more inhibitory to RNA synthesis than to protein synthesis in the same organism (Hurwitz et al., 1962). In both of these papers, the authors mention that some of their results might be explained by supposing that not all mRNA is short-lived. Very recently, Leive (1965) showed that, in \(E.\ coli\) made sensitive to actinomycin D by treatment with ethylenediaminetetraacetate, \(\beta\)-galactosidase synthesis is inhibited earlier than the incorporation of \(^{14}\)C-leucine into protein. Preliminary experiments in this laboratory have suggested that it may be possible to inhibit \(\beta\)-galactosidase synthesis with actinomycin, leaving growth largely unimpaired at least for a short period. This observation, however, requires further investigation to ensure that protein synthesis is being carried on normally, and that the inhibition of \(\beta\)-galactosidase synthesis is not due to catabolite repression.

**Findings with puromycin.** When \(E.\ coli\) was exposed to puromycin for 4 min, and the puromycin was then removed and inducer added, the kinetic picture of \(\beta\)-galactosidase synthesis was indistinguishable from the control which had not been exposed to puromycin. Thus, all inhibitory actions of puromycin developing in 4 min of contact, including the general or preferential inhibition of protein synthesis, or the possible stimulation of catabolite repression, were reversed extremely rapidly on removal of the drug. Puromycin was found to stop instantly the synthesis of \(\beta\)-galactosidase protein, but when it was present only during the first 20 sec of induction it had no effect on the subsequent course of enzyme synthesis. A contact time with puromycin of only 30 sec at the end of the induction period is sufficient to obtain an inhibitory effect on subsequent protein synthesis (Fig. 17). The fact that puromycin fails to cause inhibition of enzyme synthesis when it is present only during the first 20 sec of induction, even after 30 sec of previous contact, thus leads to the conclusion that the antibiotic does not affect the initial interaction between the inducer and its receptor. Nevertheless, it is inhibitory if present during the whole of the first 3 min after addition of inducer, even though the synthesis of the protein itself and the appearance of enzyme activity is only just beginning at 3 min. This has suggested to us that puromycin exerts an inhibitory action not only on poly peptide synthesis itself (Nathans, 1964; Sells, 1964; Williamson and Schweet, 1964), but also on some other activity occurring between 20 sec and 3 min after introduction of inducer. This other activity may be the synthesis of mRNA. The greater resistance to puromycin inhibition of overall growth compared with \(\beta\)-galactosidase synthesis might then be interpreted in terms of either a greater resistance to puromycin inhibition for the DNA-dependent transcription of mRNA for proteins other than \(\beta\)-galactosidase, or a greater functional stability for these messenger molecules once they have been synthesized. These possible conclusions lend general support to the hypothesis that various classes of messenger RNA might coexist, with different biological properties.

Sells and Takahashi (1964) confirmed that puromycin inhibits the inducible formation of \(\beta\)-galactosidase in \(E.\ coli\) to a greater extent than protein synthesis. Their evidence, like ours, suggests that puromycin does not directly affect the induction process itself. They found also that the increased incorporation of labeled adenine after
addition of the inducer was not affected by the antibiotic; but they did find that in a cell-free system, primed with synthetic mRNA, puromycin was more inhibitory when added before the messenger. These authors therefore concluded that puromycin affects the attachment of mRNA to the ribosomes. If relatively stable mRNA, already attached to the ribosomes, is therefore not sensitive to puromycin, this may account for the lesser sensitivity of growth. More recently, Sells (1965) suggested that puromycin inhibition of β-galactosidase synthesis is a consequence of catabolite repression. If we accept this new interpretation of the puromycin inhibition we might conclude, from our observations on puromycin, that catabolite repression acts not by preventing the initiation of mRNA transcription, but by inhibiting the transcription itself.

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LITERATURE CITED


PAIDEN, K. 1963. Changes in the inducibility of galactokinase and β-galactosidase during in-
LIFETIME OF BACTERIAL MESSENGER RNA


