REGULATORY PROCESSES IN ULTRA SLOW GROWING MICROORGANISMS (ESCHERICHIA COLI, BACILLUS POLYMYXA)

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Abstract
In a continuous fermentor with 100% recycle, growth of Escherichia coli and Bacillus polymyxa occurs in three sharply distinct phases: an initial phase of exponential growth that terminates when the glucose level in the fermentor falls below transport saturation levels; a second phase of linear growth whose rate is dependent on the rate of glucose provision to the fermentor, but whose length is fixed and independent of growth rate; and a third phase of slower glucose dependent linear growth. The specific growth rate falls in phase 2 and 3 while the mass doubling time lengthens, eventually reaching hundreds of hours. Guanosine 5-diphosphate 3-diphosphate (ppGpp) accumulation commences at the beginning of phase 2 in E. coli, reaching a maximum at the start of phase 3, concurrent with the slowing of RNA synthesis. This final growth stage then is an indefinitely prolonged state of regulation by the stringent response. The length of growth phase 2 was changed substantially by mutations in the relA, relX, and spoT loci, in ways which are exactly predictable from their effects on ppGpp synthesis, as phase 2 was longer for mutants with a decreased capability for ppGpp synthesis, and shorter for mutants with a decreased capability for ppGpp degradation.

In B. polymyxa, the cost of the stringent response in phase 3 is approximately 9% of the available energy, while the cost of extracellular anabolites ranges from 8 to 11% of the available energy. Both are maintenance energy costs by definition. After a carbon upshift in phase 3, the population growth rate and ppGpp concentration in B. polymyxa and E. coli proceeds to a level seen in phase 2. Fermentor levels of cAMP in E. coli rise abruptly at each phase transition, then decline throughout the phases.

The inducible DNA repair system, "SOS", can be activated in phase 3 cultures of E. coli. The degradation or turnover of proteins involved with this system, is apparently very slow, if compared with the rates of degradation of the inducible enzymes, B-galactosidase and tryptophanase. The activity of this system in phase 3 cells is interpretable as signifying the presence of a chromosome replicating fork on only 38% of the population.

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Biology, Microbiology
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REGULATORY PROCESSES IN ULTRA SLOW GROWING MICROORGANISMS

BY

Michael V. Arbige
B.S., University of Rhode Island, 1978

DISSERTATION

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in
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December, 1982
This thesis has been examined and approved.

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October 25, 1982
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ABSTRACT

REGULATORY PROCESSES IN ULTRA SLOW GROWING MICROORGANISMS

by

MICHAEL V. ARBIGE

University of New Hampshire, December, 1982

In a continuous fermentor with 100% recycle, growth of *Escherichia coli* and *Bacillus polymyxa* occurs in three sharply distinct phases: an initial phase of exponential growth that terminates when the glucose level in the fermentor falls below transport saturation levels; a second phase of linear growth whose rate is dependent on the rate of glucose provision to the fermentor, but whose length is fixed and independent of growth rate; and a third phase of slower glucose dependent linear growth. The specific growth rate falls in phase 2 and 3 while the mass doubling time lengthen, eventually reaching hundreds of hours. Guanosine 5-diphosphate 3-diphosphate (ppGpp) accumulation commences at the beginning of phase 2 in *E. coli*, reaching a maximum at the start of phase 3, concurrent with the slowing of RNA synthesis. This final growth stage then is an indefinitely prolonged state of regulation by the stringent response. The length of growth phase 2 was changed substantially by
mutations in the relA, relX, and spoT loci, in ways which are exactly predictable from their effects on ppGpp synthesis, as phase 2 was longer for mutants with a decreased capability for ppGpp synthesis, and shorter for mutants with a decreased capability for ppGpp degradation.

In *B. polymyxa*, the cost of the stringent response in phase 3 is approximately 9% of the available energy, while the cost of extracellular anabolites ranges from 8 to 11% of the available energy. Both are maintenance energy costs by definition. After a carbon upshift in phase 3, the population growth rate and ppGpp concentration in *B. polymyxa* and *E. coli* proceeds to a level seen in phase 2. Fermentor levels of cAMP in *E. coli* rise abruptly at each phase transition, then decline throughout the phases.

The inducible DNA repair system, "SOS", can be activated in phase 3 cultures of *E. coli*. The degradation or turnover of proteins involved with this system, is apparently very slow, if compared with the rates of degradation of the inducible enzymes, B-galactosidase and tryptophanase. The activity of this system in phase 3 cells is interpretable as signifying the presence of a chromosome replicating fork on only 38% of the population.
LITERATURE REVIEW

Microbial Growth and Maintenance Energy

Microorganisms, in general, have an enormous growth rate potential. Under ideal conditions, the organism, *Escherichia coli* can double its mass within twenty minutes. If a population of *E. coli* cells each weighing approximately 0.2 pg was to continue growing at this rate for as little as three days, the mass of organisms produced would be equal to about 1000 times the mass of the earth. Microorganisms do not persist at these growth rates in nature, due to a variety of growth limiting conditions, i.e. nutrient fluctuation, temperature, pH, toxic substances, and a wide array of predators. The most important of these growth constraining factors is the availability of nutrient substances, as heterotrophic organisms need to process approximately 2 grams of carbon substances, (i.e. glucose), to manufacture one gram of biomass (Tempest and Neijssel, 1981). In light of these observations, it is not unreasonable to expect that most organisms in a natural environment must cope with nutrient energy limitation as a common environmental occurrence, and adjust their metabolic machinery to handle these growth limiting situations.

It has for some time been realized that growing bacteria must divide their available metabolic energy between two basic requirements: 1) the requirement of fabrication that produce an increase in cell biomass; and 2) the requirement for reactions that may be essential for the maintenance of viability and integrity, but...
generate no increase in biomass. This latter requirement has been commonly referred to as the maintenance energy demand (Pirt, 1965).

Experimental evidence for the existence of maintenance energy did not appear until continuous culture techniques were developed (Herbert, 1958). Herbert found through chemostat experiments that when he extrapolated a plot of turbidity versus specific growth rate, the line did not follow the predicted curve. Instead, at low specific growth rates, the cell yield was less than expected from a theoretical calculation of specific growth rate and cell yield. Thus, evidence was provided for the concept of maintenance energy. McGrew and Mallette (1962) verified the maintenance energy concept in batch culture by extrapolation to zero growth plots of growth versus substrate concentration. The authors found a level of substrate, that when added to a bacterial culture at regular intervals, failed to increase the turbidity or change the viability of the culture. The absence of change in this bacterial population indicated that exogenous energy was metabolized without growth substantiating that maintenance energy did exist.

Some maintenance processes postulated by Stouthamer (1979) include the turnover of cell constituents, maintaining a pH gradient, ionic composition, and the preservation of pools of intracellular metabolites against concentration gradients. The author delineates that the amount of energy diverted to growth or maintenance depends greatly on the nature of the organisms' metabolism and on the manner in which it is cultured.
An equation relating the molar growth yield and the specific growth rate has been presented by Pirt (1965):

$$\frac{1}{Y} = \frac{m_c}{\mu} + \frac{1}{Y_{\text{max}}},$$

(1)

where $Y$ is the molar growth yield, expressed in g dry cell mass per mol energy substrate dissimilated; $\mu$ is the specific growth rate, expressed in increments in dry cell mass per increment in time per g dry cell mass; $m_c$ is the slope when equation (1) is plotted with the dimensions mol energy substrate dissimilated per g biomass per h; and $1/Y_{\text{max}}$ is the intercept of such a plot. The slope, $m_c$, is the maintenance coefficient and refers to the fraction of energy used which does not produce an increase in biomass. This energy fraction has commonly been referred to as the maintenance energy demand, and contains a wide array of ATP-consuming reactions serving many physiological roles (Stouthamer, 1979). Additionally, equation (1) assumes that the maintenance coefficient is constant at all growth rates, but evidence contradictory to this is quite strong (Neljssel and Tempest, 1976; DeVries et al., 1970; Stouthamer and Bettenhausen, 1975).

Also, predictions based on Pirt's analysis are not satisfied when the organism, *Escherichia coli*, is grown in a fermentor with 100% recycle (Chesbro et al., 1979; Arbige and Chesbro, 1982). In this system, when the cells glucose transport system becomes undersaturated, the maintenance energy demand becomes constant and independent of the specific growth rate, $\mu$, of the biomass, $X$, and
of the glucose provision rate, GPR (μmol glucose per h). After a further interval of growth that is invariant in length for a given strain, the apparent maintenance demand suddenly increases at some specific μ, by an amount that is a function of GPR, and remains constant at this new level for an undetermined amount of time. The maintenance energy demand then is independent of μ, X, and GPR during the stages of linear growth, and is dependent on μ only during the transition of these linear phases of growth.

**Fundamentals of the Recycling Fermentor**

Chesbro et al. (1979) described the construction of a chemostat with a 100% biomass feedback to study glucose-limited behavior of *E. coli* B. The theoretical basis underlying the apparatus, and many of its applications have been extensively examined by Evans (1978) and Elfert (1979).

The basic design of the recycling fermentor resembles partially what Pirt and Kurowski (1970) have described as a chemostat with feedback and the dialysis apparatus of Schultz and Gerhardt (1969). In all these systems, theory predicts that growth would asymptotically approach an ultimate biomass which would be determined by both the GPR and the organisms' maintenance energy requirement. The population should then stabilize at a point where all metabolic energy is used for retention of viability, with no energy available for further biomass increase. This theoretical "maintenance state"
(figure 1) was not experimentally observed when *E. coli* was grown in a recycling fermentor. The growth rate never approached zero, as the cells apparently always partitioned some of the incoming energy to reactions that fabricate cell biomass. Instead, growth occurred in three sharply distinct phases (Chesbro, 1979) (figure 2). The initial phase was a period of batch type growth reaching exponential rates which terminated suddenly when the glucose concentration in the fermentor fell below the cells' transport level. The second phase in which the growth rate was a linear function of GPR was a period of slower but balanced growth, at least in respect to rates of RNA, DNA, and protein syntheses. This phase was fixed at a strain-specific length and was then succeeded by phase 3 in which the growth rate abruptly slows to a fraction of the phase 2 rate. In this phase, growth became unbalanced as the rate of RNA synthesis was depressed more than that of the other macromolecules (Evans, 1979).

In this system, the growth rate, i.e. the rate of mass increase, \( dx/dt \), is constant and positive in phases 2 and 3, while the cell mass, \( X \), increases continuously. Therefore, the specific growth rate, \( \mu, x^{-1} dx/dt^{-1} \), decreases continuously within both phases, with an abrupt decline in its own rate between phases 2 and 3. During this final period of growth, the doubling time can surpass the 1000 hour range, which is characteristic of senescent or quiescent eukaryotic cells. Thus, the recycling fermentor allows concise investigation of cellular processes in cells with very long mass
Figure 1: Theoretical maintenance curve.

Theoretical prediction of growth in a population where all metabolic energy is eventually used for maintaining culture viability.
Theoretical "Maintenance State"
Figure 2: Three phase growth pattern of *Escherichia coli* B.

Three phase growth pattern observed by Evans (1978) when *E. coli* B was cultured in a recycling fermentor.
doubling times occurring along a protracted gradient of continuously decreasing specific growth rates (Chesbro et al., 1979; Arbige and Chesbro, 1982).

The Bacterial Stringent Response

Bacterial stringent regulation is the response of bacterial cells to either amino acid or energy starvation in which cell biosyntheses are differentially affected by the special nucleotide, guanosine-3',5'-diphosphate (ppGpp), produced under these circumstances. It is the single most powerful regulatory effector known in bacterial cells, depressing the synthesis of protein and rRNA while augmenting the synthesis of some species of mRNA (Gallant, 1979).

The stringent response was first described more than thirty years ago by Sands and Roberts (1952), who showed that the rate of RNA accumulation in E. coli was drastically reduced when the availability of any amino acyl-tRNA species become limiting for protein synthesis. This can occur either through an amino acid deficiency or the inactivation of an amino acyl-tRNA synthetase. This decrease in RNA accumulation is considered the "hallmark" of the stringent response (Nierlich, 1978). Nierlich emphasizes that control over synthesis of the RNA components of the protein synthesizing system, primarily the stable species of RNA, is fundamental to the regulation of growth. Stent and Brenner (1961)
first described mutants of *E. coli* which do not curtail their RNA synthesis when deprived of an amino acyl-tRNA species, and they termed them "relaxed" cells as opposed to their stringent counterparts. These mutations and most mutants isolated since, map specifically in the relA locus (Fill and Frlesen, 1968).

Many adjustments occur in cells undergoing the stringent response, among which include: 1) restricted membrane transport of various exogenous precursors; and 2) reduced nucleotide, carbohydrate, glycolytic intermediate, lipid, fatty acid, polyamine, and peptidoglycan syntheses (Cashel, 1975; Gallant and Lazzarinl, 1976).

Since both metabolic and transcriptional patterns were affected by this response, a search ensued for low molecular weight mediator(s) of the stringent response. This inquiry revealed two unusual guanine nucleotides, first termed magic spots (MS) 1 and 2 (Cashel and Gallant, 1969). These compounds have subsequently been identified as guanosine-5'-diphosphate-3'-diphosphate (ppGpp) and guanosine-5'-triphosphate-3'-diphosphate (pppGpp), respectively (Sy and Lipmann, 1973).

Presently, the accepted outline scheme for synthesis and degradation of ppGpp proceeds along two routes, and appears to be regulated by at least four gene loci (figure 3) (Gallant, 1979). The relA gene product synthesizes pppGpp, a precursor of ppGpp, through a ribosome-bound ATP:GTP pyrophosphate transferase whose activity
requires the presence of mRNA and uncharged, codon-specific tRNA.
These requirements link this reaction to amino acid deficiency.
Carbon source depletion would deplete the cell's amino acid pool,
therefore, the relA gene product is coupled indirectly to the carbon
source supply. The second system involving the relX gene product,
and the relS locus (Engel, et al., 1979) stimulates ppGpp production
directly in response to a carbon/energy deficiency. pppGpp is not an
intermediate in this second system. Other mutations conferring a
relaxed phenotype have been isolated, but have subsequently been
shown to have epistatic effects on relA. One mutation exists in the
relC locus, which codes for ribosomal protein L11, and would, in
turn, reduce stringent factor activity on the ribosome (Fill et al.,
1977). The other mutation occurs in the relB locus which leads to
the production of an endogenous ribosome inhibitor during amino acid
starvation (Mosteller, 1978).

Regardless of the route by which ppGpp is synthesized, it is
degraded by the spoT gene product, guanosine-3',5'-bis-(diphosphate)
-3'- (pyrophosphohydrolase), an enzyme which is also ribosome-
associated (Fill et al., 1977; Richter et al., 1979), but whose
activity can be dampened by uncharged tRNA (Richter, 1980). The
enzyme also requires the existence of an intact trans membrane proton
gradient for its activity (DeBoer et al., 1975; Tetu et al., 1980).
Glucose exhaustion or uncoupling of oxidative phosphorylation, which
more than likely collapses the proton gradient, both cause an
Figure 3: Scheme for the synthesis and degradation of ppGpp.

Scheme for synthesis of ppGpp proposed by Gallant (1979). Solid lines refer to reactions that have been demonstrated \textit{in vitro}, while the dotted lines indicate postulated reactions that have yet to be demonstrated \textit{in vitro}. Single-headed arrows reflect irreversible hydrolytic reactions, and double-headed arrows indicate reversible reactions.
Figure 3
Instantaneous decrease in ppGpp degradation (DeBoer et al., 1975; Tetu et al., 1980). A decrease in ppGppase activity elevates the level of ppGpp concentrations in both amino acid and energy limiting situations (Belitsky and Shakulov, 1982). Levels of ppGpp have been shown to approach 1 mM intracellular concentration during the stringent response, which is very close to the level of ATP in the cell (Gallant, 1979).

**Range of Organisms Under Stringent Control**

Due to the extensive genetic library that exists for the organism, _Escherichia coli_, most of the studies concerning the stringent response have been performed with this bacterium. A diverse range of additional bacteria including _Bacillus subtilis_ and _Salmonella typhimurium_ have been studied and most investigations have demonstrated the physiological adaptations of stringent control (Swanton and Edlin, 1972; Stephens, Arts and Ames, 1975). Hence, it is likely that much of the information gathered on _E. coli_ is applicable, at least in a broad sense, to most of the prokaryotic world (Cashel, 1975).

Probing for these nucleotides in eukaryotic cells has revealed a plethora of contradictory evidence (Silverman and Atherly, 1979). In almost every instance where ppGpp is demonstrated to accumulate in eukaryotes, there is evidence to refute it. The general sentiment among investigators on this topic is that some eukaryotic cells may
synthesize ppGpp in response to various environmental stresses, but there is probably very little regulatory significance associated with this nucleotide (Silverman et al., 1979).

The Bacterial Cell Cycle

The relationship between chromosome synthesis and cell division during the bacterial cell cycle has been investigated and reviewed with increasing activity over the past decade (Donachie et al., 1976; Helmhstetter and Pierucci, 1976; Shockman et al., 1974; Tang and Helmhstetter, 1980). This area of research, like too many others, suffers from the common syndrome of overgeneralization. The parameter in this field which has produced the most conflicting interpretations is the effect of growth rates on the cell cycle (Sloan and Urban, 1976; Loeb et al., 1978). One of the current presentations of the cell cycle follows the I + C + D model. C is the time required for replication of the bacterial genome from the origin to the terminus; D is the time between the end of chromosome replication and cell division; and I is the period, if any, between the end of D and initiation of C (Cooper and Helmhstetter, 1968). These periods do, however, overlap each other under some circumstances. This model has been developed to account for cell cycle patterns in bacterial cells growing with doubling times between approximately 20 and 120 min, with the latter part of this scale (doubling times of 60 min and higher) considered to be typical of slowly growing cells.
*Escherichia coli* and *Salmonella typhimurium* cells growing with doubling times greater than 60 min have been reported to differ considerably from cells grown with doubling times of 40 min or less, as the slower growing cells show periods in the cell cycle where no DNA synthesis occurs (Cooper and Helmstetter, 1968). The number of chromosomes per cell also varies between slow and fast growing cells, as *E. coli* 15 T− has been shown to contain up to and including four chromosomes in cells growing with 20 min doubling times, two chromosomes at 40 min doubling times, and one chromosome in cells with 120 min doubling times (Lark, 1966; Cooper and Helmstetter, 1968). These reports and reports that the length of the D period is similar in fast and slow growing cells (Kubitschek, 1974) have been disputed by others (Sloan and Urban, 1976; Holmes et al., 1980).

In the present evaluation of the microbial cell cycle determining the exact link between chromosome replication, cell elongation, and septation for all growth situations poses a dilemma. This correlation between chromosome replication and cell elongation using a range of relatively fast growing cells has been summarized by Pierucci (1978), who postulated the following: 1) new zones of envelope growth are stimulated simultaneously with the initiation of C; 2) each zone remains active until the completion of C and D; and 3) the rate of envelope elongation is constant and totally independent of growth rate. This model, which corresponds well with those of Begg and Donachie (1978), Shockman et al. (1974), Tang and
Helmstetter (1980) as well as others, predicts that if a population of cells growing exponentially is exposed to a nutrient upshift, there will be no immediate acceleration in cell division. The mechanism of envelope growth and extension would be locked at the pre-shift rate until new zones of growth are activated by the initiation of chromosome replication. This phenomenon has been termed rate maintenance (Kjeldgaard et al., 1958) and has been observed repeatedly over the years (Helmstetter et al., 1965; Cooper, 1969). Sloan and Urban (1976) reported that cell division did accelerate immediately following a nutrient upshift of E. coli strains B/r, K12, or 15T- growing with doubling times of two hours or longer. The authors insisted that rate maintenance, although evident at faster growth rates, did not apply to these strains at longer doubling times. Pierucci (1978) was unable to confirm this, but employed an upshift from a doubling time of 120 to 40 min, while Sloan and Urban upshifted their cells from 120 to 60 or 30 min. Thus, Pierucci did not repeat the experiment exactly.

When E. coli cells grown in a recycling fermentor, with doubling times in excess of 100 h are subjected to a four-fold GPR upshift in phase 3, the observations were similar to those of Sloan and Urban (Evans, 1978). Cell division rates were immediately stimulated, as were rates of biomass, RNA, and protein accumulation. Sloan and Urban felt that a plausible explanation for their observations was that cells growing slowly completed the period between initiation of chromosome replication and cell division (C and D period) more
rapidly than predicted by the growth rate, and such cells were "primed" for division.

"SOS" Repair System

DNA damage and its repair are significant factors influencing cell aging, viability, and susceptibility to adverse conditions. Although prokaryotic cells differ in their rates of DNA synthesis and repair (Kornberg, 1980), analogies between gene repair mechanisms in bacterial and mammalian systems have been consistently presented (Smith, 1976). When bacterial cells are grown in a recycling fermentor, some of these discrepancies are eradicated (Chesbro et al., 1979), and this system would therefore provide a more useful model for studies of this nature. The fermentor slows the bacterial cell cycle by over 100-fold, thus, causing it to resemble more closely the cell cycle of eukaryotic cells. At these slow rates of biomass and DNA synthesis, it would seem obvious that events occurring at the molecular level in gene replication would be easier to detect and manipulate. Additionally interesting is that at these low synthetic rates, the proteolytic dependent systems of DNA repair might well be impaired, as Evans (1978) discovered that proteolysis is at a reduced level in phase 3 cells, particularly the error-prone SOS system, responsible for much of the ultraviolet and chemical mutagenesis in E. coli, may provide investigators with some information on cell aging.
The SOS response is the primary route by which premutagenic lesions are fixed into the bacterial chromosome as mutations. Ultraviolet light exposure precipitates many events in the bacterial cell including lysogenic induction, synthesis of DNA repair enzymes, interruption of DNA replication, filamentation (i.e. no cell division), increased rates of mutagenesis, and an increased rate of synthesis of a 38,000 MW protein (Witkin, 1976). The interrelated events of the SOS response and their correlation with various gene products have been reviewed recently by Flanders (1981). Workers have revealed that in undamaged cells, the chromosome repair systems are repressed by the LexA gene product, a protein with a molecular weight of 24,000 (Mount et al., 1972). However, small amounts of the uvr genes' repair enzymes are omnipresent to handle minor repairs. In the event of extensive exposure to UV irradiation or other DNA damaging agents, i.e. mitomycin C, nalidixic acid, bleomycin, or MMNG, a substantial number of pyrimidine dimers are formed in the bacterial chromosome which eventually influence formation of post-replication gaps (Rupp and Flanders, 1968). This subsequently triggers RecA protein, the 38,000 MW product of the recA gene, already existing at low intracellular levels, to bind to the single strand DNA opposite the gaps. The protein-DNA complex stimulates the proteolytic activity of RecA (Roberts et al., 1977) which catalyzes an entire series of changes in the cell. The "activated" RecA protein cleaves the LexA repressor, allowing expression of the recA gene, the excision repair enzymes,
and in turn, its own production (Mount et al., 1972). The protease also degrades a repressor sustaining bacteriophage lambda in the prophage state. This activity allows the synthesis of the virus particle, and the eventual destruction of the cells (Roberts et al., 1977). Active RecA protein, however, has been proven to cleave the LexA repressor more rapidly than it cleaves lambda repressor, suggesting that LexA is its primary substrate (Little et al., 1980). Additionally, RecA protein is involved with interrupting DNA replication and cell division, as it acts on the SulA and B genes and their products which are responsible for suppressing filamentous growth and allowing normal cell division (George et al., 1975). Once recA is released, massive amounts of RecA protein are synthesized, reaching levels as high as three to four percent of the total cell protein (Gudes and Pardee, 1976). The newly-synthesized protein binds to any single-stranded DNA remaining, and then to adjacent double-stranded regions, preventing the stabilization of any newly-manufactured LexA protein (Little et al., 1980). The regulated genes continue functioning until the single-stranded gaps are repaired. When post-replication repair is complete, RecA becomes inactivated, LexA protein is no longer cleaved, and the cell returns to the uninduced state (Flanders, 1981) (figure 4). No portion of the SOS response is expressed in cells carrying either a recA or a lexA mutation, but all situations are expressed constitutively at 42°C in cells exhibiting a tlf mutation (Castellazzi et al., 1972).
Scheme for the regulatory system based on lex A and rec A produced by Flanders (1981). In the uninduced state, lex A repressor binds to operators of many genes, preventing the synthesis of mRNA and protein at a low level. Damage to DNA, which produces post-replication gaps, activates the "SOS" response. Rec A protein then binds to single-stranded DNA opposite a gap, triggering its proteolytic activity. Lex A repressor is cleaved and all the controlled genes are switched on, until DNA repair is completed.
Figure 4
The recA protein is considered to be quite stable in cultures of *E. coli* B/r and *E. coli* B growing exponentially (Gudes and Pardee, 1976). To verify this hypothesis, the authors labeled cultures with $^{35}$S-methionine during the inhibition of DNA synthesis by nalidixic acid, and then chased with non-radioactive methionine. Gel electrophoresis and densitometry revealed that the labeled protein was still present after one generation of growth without nalidixic acid. The authors concluded that the protein must be diluted out by cell growth, rather than degraded by proteases. If this is indeed the case, than any RecA produced in a phase 3 culture of *E. coli* should be stable for up to 1000 h.

The SOS response promotes DNA repair by increasing the level of excision repair enzymes, but Witkin (1976) also suggests that a special "error-prone" type of DNA repair is associated with the increased mutagenesis in these induced cells. Increased levels of an error-prone DNA polymerase have recently been found in SOS induced cells (Flanders, 1981), but the mechanisms involved in this phenomenon have not yet been worked out.

**Inducible Enzyme Production and Degradation in the Recycling Fermentor**

Evans (1978) studied the induction and decay of B-galactosidase activity in the fermentor with the organism, *E. coli* B. When these cells were continuously induced in phase 3 with
Isopropylthiogalactoside (IPTG), B-galactosidase levels rose, and became constant for at least 50 h, with twelve to fifteen percent of all cell proteins becoming lac operon proteins. During this induction, the total rate of protein synthesis was depressed. When cells were induced in phase 2 with a pulse of lactose, B-galactosidase activity peaked and then decayed rapidly until about thirty percent of the activity had disappeared. The rate of degradation then decreased markedly as the culture entered phase 3, and enzyme activity disappeared thereafter at a low constant rate. When a four-fold increase in carbon energy rate was imposed on the cells, the remaining enzyme activity disappeared rapidly. Evans concluded through these studies and others concerning $^{14}$C-labeled proteins that macromolecular turnover decreases as the cells proceed from phase 2 to phase 3.

It seemed useful to have a second inducible enzyme as a further marker of general protein degradation processes in these slow-growing cells prior to examining the decay of the RecA protein during the SOS response. The enzyme, tryptophanase suggested itself for these investigations. Tryptophanase is a 281,000 MW protein which is composed of five subunits (Snell, 1975). The mechanisms and kinetics of induction of this enzyme in E. coli have been shown to be very similar to those of B-galactosidase (Bilezikan et al., 1966). Knowledge of the reaction catalyzed by this enzyme was first summarized by Wood et al. (1947) and is shown in the scheme below,
where the inducer, tryptophan, is degraded by the enzyme and cofactors indicated to produce stoichiometric amounts of indole, pyruvate and ammonia:

\[
\text{TPase} \\
L\text{-tryptophan} + H_2O \rightarrow \text{Indole} + \text{pyruvate} + \text{NH}_3
\]

\[\text{pyridoxal-5'-phosphate, } \text{NH}_4\]

Tryptophanase has been found in a wide variety of physiologically and morphologically diverse bacteria (Table 1). It is present in small amounts in cells grown in the absence of tryptophan, is repressed in cells grown in the presence of glucose or other rapidly metabolized carbon sources (Boyd and Lichstein, 1955), and can reach levels in cells approaching ten percent of the soluble cell protein in fully induced cultures (Snell, 1975). This enzyme has been studied by only a handful of researchers over the past decade, and little or no data as to its distribution and longevity in the bacterial cell exist.

The Production of Cyclic Nucleotides in Carbon-Limited Cells

The phosphotransferase system (PTS), the glucose transport system in \textit{E. coli}, becomes undersaturated with substrate as cells grown in the recycling fermentor enter phase 2 (Chesbro et al., 1979). At this point, \(\beta\)-galactosidase becomes fully inducible (Evans, 1978) implicating the production of cAMP (Pastan and Adhya, 1976). The impact of cAMP production in these energy depleted cells may be of grave importance to cellular function and integrity and, therefore, renders itself worthy of investigation.
TABLE 1: The distribution of tryptophanase (TPase) in different bacterial genera (Snell, 1975)

<table>
<thead>
<tr>
<th>Genus</th>
<th>Number of strains tested</th>
<th>Number of strains containing TPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetobacter</td>
<td>79</td>
<td>0</td>
</tr>
<tr>
<td>Achromobacter</td>
<td>61</td>
<td>0</td>
</tr>
<tr>
<td>Aeromonas</td>
<td>8</td>
<td>$^a$</td>
</tr>
<tr>
<td>Agrobacterium</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>Azotobacter</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Bacillus</td>
<td>?</td>
<td>$^b$</td>
</tr>
<tr>
<td>Bacteroides sp.</td>
<td>?</td>
<td>1</td>
</tr>
<tr>
<td>Clostridium</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>?</td>
<td>$^c$</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>65</td>
<td>$^{12d}$</td>
</tr>
<tr>
<td>Erwinia</td>
<td>131</td>
<td>$^e$</td>
</tr>
<tr>
<td>Escherichia</td>
<td>44</td>
<td>$^f$</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>89</td>
<td>0</td>
</tr>
<tr>
<td>Kluyvera</td>
<td>5</td>
<td>$^g$</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>?</td>
<td>$^h$</td>
</tr>
<tr>
<td>Mycoplana</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Paracolobactrum</td>
<td>?</td>
<td>$^i$</td>
</tr>
</tbody>
</table>

(continued on next page ...
<table>
<thead>
<tr>
<th>Genus</th>
<th>Number of strains tested</th>
<th>Number of strains containing TPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasteurella</td>
<td>?</td>
<td>1</td>
</tr>
<tr>
<td>Photobacterium</td>
<td>?</td>
<td>1</td>
</tr>
<tr>
<td>Proteus</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>475</td>
<td>0</td>
</tr>
<tr>
<td>Rhizobium</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Salmonella</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Serratia</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>Sphaerophorus</td>
<td>?</td>
<td>1</td>
</tr>
<tr>
<td>Vibrio</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Xanthomonas</td>
<td>19</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>aAeromonas liquefaciens.</td>
</tr>
<tr>
<td>bBacillus alvei.</td>
</tr>
<tr>
<td>cCorynebacterium acnes.</td>
</tr>
<tr>
<td>dEnterobacter aerogenes (two strains) and E. liquefaciens (ten strains).</td>
</tr>
<tr>
<td>eErwinia herbicola (two strains) and E. carotovora (six strains).</td>
</tr>
<tr>
<td>fEscherichia coli (eight strains); numerous additional strains investigated by other authors also produce TPase.</td>
</tr>
<tr>
<td>gKluyvera citrophila (two strains).</td>
</tr>
<tr>
<td>hMicrococcus aerogenes.</td>
</tr>
<tr>
<td>iParacolobactrum coliforme.</td>
</tr>
</tbody>
</table>
In addition to the well-known role of cAMP and catabolite repressor protein, CRP, a cAMP receptor protein, in the initiation of transcription of inducible catabolic operons (Pastan and Perlman, 1970), the cAMP-CRP complex has been shown to affect many additional functions in *E. coli*. These functions include lysogeny by bacteriophage (Pastan and Adhya, 1976), replication of plasmids (Katz et al., 1973), envelope properties including regulation of synthesis of flagella, fimbriae, pilus, fluidity of membrane, sensitivity to detergents, composition of proteins in outer membranes, and sensitivity to antibiotics (for review, see Botsford, 1981).

Like guanosine-3',5'-bispyrophosphate (ppGpp), the intracellular levels of cAMP in *E. coli* are inversely proportional to the growth rates. Both nucleotides are involved in a number of control activities, and accumulate when cells are starved for glucose or when shifted from glucose to a poorer carbon source. In fact, accumulation of ppGpp and cAMP are both affected by mutations in the relA locus (Braedt and Gallant, 1976), suggesting some correlation between the two compounds. When cells are starved for an amino acid though, only ppGpp accumulates, while cAMP synthesis is not affected (Botsford and Drexler, 1978).
MATERIALS AND METHODS

Strains and Cultural Conditions

All *Escherichia coli* strains (Table 2) were maintained on Davis and Mingioli (1950) basal medium (DMM) agar slants containing 1.25 mg ml$^{-1}$ glucose with appropriate amino acids added at the level of 20 μg ml$^{-1}$ for the growth of auxotrophic strains. *Bacillus polymyxa* strains (Table 2) were maintained on nutrient agar slants. All cultures were periodically restarted from stocks frozen at -70°C in tryptase soy broth containing 0.3% yeast extract and 10% dimethyl sulfoxide (DMSO).

The basal medium in all fermentor experiments with *E. coli* strains except those involving $^{32}$P, was DMM, a known glucose concentration, 10 μg ml$^{-1}$ dithiothreitol (Chesbro et al., 1979) (DTT), and appropriate amino acid supplementation at a concentration of 20 μg ml$^{-1}$. The medium was prepared and autoclaved in 10 or 18 liter batches. The medium used in all fermentor experiments with *B. polymyxa* was a modified Katznelson and Lochhead (1944) minimal medium containing in g or ml$^{-1}$: glucose, 1.5; MgSO$_4$·7H$_2$O, 0.2; NaCl, 0.1; CaCl$_2$, 0.1; FeSO$_4$·7H$_2$O, 0.01; ZnSO$_4$, 0.01; MnSO$_4$·H$_2$O, 0.0075; 40 ml of a 0.5 M K$_2$HPO$_4$ solution, pH 7.5. These components were boiled together for 5 min and then filtered to remove precipitates. After filtration, biotin was added, 0.5 μg l$^{-1}$, and 0.25% (v/v) of a
TABLE 2. Strains of *Escherichia coli*, *Bacillus polymyxa*, and *Yersinia pestis* utilized for recycling fermentor studies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Genotype/Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. polymyxa</em></td>
<td>H. Paulus</td>
<td>met</td>
</tr>
<tr>
<td>Pfizer2459</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. polymyxa</em></td>
<td>H. Paulus</td>
<td>an asporogenous slime-less derivative of Pfizer2459</td>
</tr>
<tr>
<td>ATCC25901</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> NF859</td>
<td>J. Gallant</td>
<td>argA, metB</td>
</tr>
<tr>
<td><em>E. coli</em> NF859X</td>
<td>J. Gallant</td>
<td>argA, metB, relA1</td>
</tr>
<tr>
<td><em>E. coli</em> NF162</td>
<td>J. Gallant</td>
<td>argA, metB, relA1, spoT</td>
</tr>
<tr>
<td><em>E. coli</em> NF161</td>
<td>J. Gallant</td>
<td>argA, metB, spoT</td>
</tr>
<tr>
<td><em>E. coli</em> NF1035</td>
<td>J. Gallant</td>
<td>argA, metB, relA1, relX</td>
</tr>
<tr>
<td><em>E. coli</em> H10407</td>
<td>S. Falkow</td>
<td>ent⁺</td>
</tr>
<tr>
<td><em>E. coli</em> AB1157</td>
<td>D. Mount</td>
<td>pro, arg, thr, leu, his, thi</td>
</tr>
<tr>
<td><em>E. coli</em> B</td>
<td>laboratory strain</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> NF161-λ</td>
<td>this study</td>
<td>argA, metB, spoT, λ</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC33311</td>
<td>ATCC</td>
<td>lacZ gene fused to operon under λ repressor control</td>
</tr>
<tr>
<td><em>Y. pestis</em> [CAV51F]</td>
<td>R. Zsigray</td>
<td>--</td>
</tr>
<tr>
<td><em>E. coli</em> CSH22</td>
<td>laboratory strain</td>
<td>λ-sensitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
vitamin-free enzymatically hydrolyzed casein solution (ICN, Nutritional Biochemicals, Cleveland, OH). The solution was cooled, adjusted to the desired pH, autoclaved, recooled, and a sterile solution of glucose containing 10 μg ml⁻¹ DTT added aseptically.

For the growth of Yersinia pestis, a modified MASGF medium was used (Table 3) (Burrows and Gillett, 1966).

Recycling Fermentor Operation

The recycling fermentor has been described previously (Chesbro et al., 1979; Evans, 1978) (Figure 5). Briefly, sterile, oxygen-free medium limiting in a specific nutrient is added to the reaction vessel at a controlled rate. All the cells of a growing population are retained in the growth chamber by recycling the chamber's contents (450 ml) across a partitioning Amicon TCIR recycling unit containing a 0.2 μ polycarbonate filter (Nucleopore Inc., Pleasanton, CA). At this point, the spent medium is drawn off at the same rate the new medium is being added and the microbial population returned to the reaction vessel. Thus, during each succeeding interval of operation a constant amount of the limiting nutrient, i.e. glucose, will be added to a continuously increasing population of cells resulting in a continuously decreasing amount of the limiting nutrient per unit of cell mass. The glucose provision rate, GPR, is the product of the substrate concentration of the medium (SR) multiplied by the rate at which medium flows into the fermentor (FR).
TABLE 3. Modified MASGF medium utilized for culture of

*Yersinia pestis*

<table>
<thead>
<tr>
<th>Compound</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_2HPO_4$</td>
<td>10.5</td>
</tr>
<tr>
<td>$KH_2PO_4$</td>
<td>4.5</td>
</tr>
<tr>
<td>$(NH_4)_2SO_4$</td>
<td>1.0</td>
</tr>
<tr>
<td>Na citrate</td>
<td>0.5</td>
</tr>
<tr>
<td>$\text{MgSO}_4\cdot7H_2O$</td>
<td>0.62</td>
</tr>
<tr>
<td>$Na_2S_2O_5$</td>
<td>0.01</td>
</tr>
<tr>
<td>Acid hydrolyzed casein</td>
<td>2.0</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>0.04</td>
</tr>
<tr>
<td>thiamine (1 μmol)</td>
<td>0.000337</td>
</tr>
<tr>
<td>Ca pantothenate (1 μmol)</td>
<td>0.000477</td>
</tr>
<tr>
<td>biotin (0.01 μmol)</td>
<td>0.0002443</td>
</tr>
<tr>
<td>yeast extract</td>
<td>0.3</td>
</tr>
<tr>
<td>glucose</td>
<td>1.25</td>
</tr>
</tbody>
</table>

autoclave separately
Figure 5: Diagram of maintenance energy fermentor.

Schematic diagram of maintenance energy fermentor constructed by Chesbro et al. (1979). The parameters are: fermentor volume ($V_F$), substrate concentration in medium reservoir ($S_R$), substrate concentration in fermentor ($S_F$), cell mass ($X$), flow rate of medium into fermentor ($F_R$), and flow rate from the filter apparatus ($F_S$).
Figure 5
Upon start up, the fermentor is seeded with ca. 30 ml of a 10 h anaerobic culture. The reaction vessel is maintained at 30°C, and the pH held constant by the addition of 2N NaOH.

**Cellular Assays**

At set intervals, samples were withdrawn from the fermentor and analyzed. Cell dry weights were determined in triplicate by filtering samples onto pre-weighed, dried, 0.2 μm Nuclepore carbonate filters (25 mm diameter). The filters were rinsed three times with prefiltered 0.1% (v/v) formaldehyde dried at 60°C for 48 h and weighed on a Cahn electrobalance. Klett readings were performed on a Klett-Summerson instrument with a #54 filter. Viable counts were determined on minimal medium in triplicate by the spread plate method. Cellular protein was determined by a modified Lowry procedure (Brunschede et al., 1977). Cells were first hydrolyzed by diluting (1:5) into 0.5 N NaOH and boiling for 5 min. The samples were then frozen for analysis at a later time. Bovine serum albumin fraction V (Fischer Scientific) was used as the standard.

The procedure of Donkersloot et al. (1972) was used for determining cellular DNA. The DNA samples were prepared by filtering cells onto a 0.2 μm polycarbonate membrane and rinsing them once with 1 ml of a saline-EDTA solution. The filtered cells were resuspended in 2 ml of 0.3 N KOH, hydrolyzed at 37°C, neutralized with 0.5 ml of 1 N HCl, and then frozen for analysis at a later date. An equal
volume of 4 μg/ml ethidium bromide was added to the samples and fluorescence was measured in a Turner III fluorometer. Calf thymus DNA (Sigma) was used as the standard.

Cellular RNA was determined by the orcinol procedure, on samples that had been frozen in 0.8% formaldehyde. After thawing, the cells were washed three times in cold 10% trichloroacetic acid (TCA), followed by heating the cells for 15 min in 5% TCA. Yeast RNA (Sigma) was used as the standard.

**Determination of ppGpp**

To four liters of modified DMM containing in g l⁻¹: K₂HPO₄, 0.087; NH₄SO₄, 1.0; Na citrate, 0.5; Bicine, 16.317 (Aldrich Chemical Co., Milwaukee, WI); MgSO₄.7H₂O, 0.1 (autoclaved separately); glucose, 3.75 (autoclaved separately); DTT, 0.01 at pH 7.0, was added 25 mCi of carrier-free ³²P-orthophosphate (New England Nuclear).

The fermentor was seeded and 2 ml samples drawn at various time intervals were made 2M in formic acid by addition of 153 μl of concentrated formic acid. Samples were frozen at -70°C, thawed, centrifuged, and the supernate dried and resuspended in 200 μl of 10% isopropanol. The suspension was neutralized and concentrated 10X. These samples were used to spot polyethyleneimine (PEI)-cellulose thin layer chromatograms (Cashel et al., 1968).

Separation of ppGpp was carried out by PEI-cellulose thin layer chromatography. The sheets were prewashed once in delonized water and dried. One dimensional separation of the nucleotides included
spotting between 2.5 and 10 µl of $^{32}$P-labeled nucleotide extracts and chromatographing in 1.5 M potassium phosphate buffer, pH 3.4 (Cashel et al., 1968). $^{32}$P-labeled nucleotides were detected by radioautography using Kodak XR-5 x-ray film exposed from 24-72 h. Spots whose mobility corresponded to authentic standards of ppGpp and GTP (PL Biochemicals Inc., Milwaukee, WI) were cut and counted in a Packard Tri-carb Scintillation Spectrophotometer.

For qualitative identification of ppGpp and other nucleotides, a two dimensional system was employed (Cashel et al., 1968). This consisted of chromatographing the formic extracts in the first dimension with 3.3 M ammonium formate + boric acid, pH adjusted to 7.0 with NH$_4$OH. Sheets were then soaked in methanol for 5 min, water for 15 min, and dried. The second dimension utilized 0.85 M KH$_2$PO$_4$, pH 3.4.

**Determination of RelA Phenotype**

The RelA phenotype was tested by the procedure described by Engel et al. (1979) which involved growing cells overnight on broth agar plates, picking cells with a toothpick to make turbid 200 µl of a Tris minimal glucose media (Kaempfer and Magasanik, 1967) containing 40 µM phosphate, 2 mg ml$^{-1}$ serine hydroxamate (Sigma), 300 µg ml$^{-1}$ L-valine (Sigma), and 10 µCi ml$^{-1}$ $^{32}$P. This mixture was incubated for one h at 37°C. 50 µl of 13 N formic acid was then added, and the solution frozen for 15 min. Ten µl was spotted on PEI-cellulose chromatography plates, developed 10 cm with 1.5 M
KH₂PO₄, pH 3.4 and exposed to x-ray film overnight.

**Arsenate Inhibition**

Five liters of modified DMM was supplemented with ca. 30 mCi of ³²P and growth in the fermentor was allowed to proceed for 61 h. 0.3 g of potassium arsenate (Sigma) was then added to the fermentor. Subsequent 2 ml samples were brought to a concentration of 2 M formic acid, frozen at -70°C, thawed, centrifuged, and supernates neutralized with 0.52 ml of 5 N NaOH, and 0.35 ml of 1.75 M K₂HPO₄. Samples were treated with charcoal by a modification of the method used by Griffin et al. (1965). One ml of supernate was batch absorbed on 25 mg Norite A activated charcoal for 30 min at 4°C. The charcoal was recovered and washed with H₂O. Nucleotides were then eluted with 250 µl of H₂O-ethanol-NH₃ (65:35:3; v/v). These samples were then used to spot PEI-cellulose thin layer chromatograms.

**Determination of Cyclic Adenosine 3',5'-Monophosphate**

The method of Braedt and Gallant (1976) was employed to measure ³²P-labeled cAMP. Formic acid extracts that were absorbed on charcoal were developed on PEI-cellulose layers in isopropanol-ammonium hydroxide-water (70:15:15; v/v). The plates were cut 6 mm behind the authentic cAMP marker (Sigma), and developed again in n-butanol-acetic acid-water (50:25:25; v/v). Spots coinciding with marker cAMP were cut and counted in a scintillation counter.

Using known amounts of titrated cAMP (New England Nuclear) in
the charcoal adsorption procedure, consistently rendered a recovery of 17% for cAMP which was used as the correction in calculating recoveries of $^{32}$P-cAMP.

**Determination of Filtrate Components**

The presence of DTT in the filtrate was found to interfere with the analysis of RNA, DNA, and protein. Therefore, each assay was corrected for this interference. The filtrate material was twice filtered over mini-columns containing Amberlite, IR-120 (H⁺) (Fischer Scientific) to reduce the salt concentration in the samples, and the column was washed with a volume of distilled water equal to the bed column.

Protein in the filtrate was determined on the treated samples by the Lowry procedure (Lowry et al., 1951), RNA by the orcinol procedure (Schneider, 1957), and DNA by the diphenylamine technique (Burton, 1956).

Glucose in the filtrate was determined by the UV method, based on the use of hexokinase and glucose-6-phosphate dehydrogenase (Sigma).

Ethanol in the filtrate was quantified by NAD reduction using ethanol dehydrogenase (Anonymous, 1977). Acetate and 2,3-butylene glycol were determined by the methods of Nelsh (1952).

For qualitative determination of non-gaseous fermentation products in the filtrate, a Perkin Elmer model 3920 gas chromatograph equipped with a hydrogen flame detector was used. The products were
separated on a stainless steel column (0.32 cm X 305 cm) packed with 10% SP-1000 (1% $\text{H}_3\text{PO}_4$) on 100/120 mesh chromosorb WAW (Supelco). Conditions used in the analysis were: carrier gas, $\text{N}_2$, flow rate 30 ml min$^{-1}$; injection port temperature, 170°C; $\text{H}_2$ flow, 50 ml min$^{-1}$; and air flow, 600 ml min$^{-1}$. Samples and standards were prepared as described in the Anaerobe Laboratory Manual (1975).

**Electron Microscopy**

Samples for thin sectioning were taken out of the fermentor and fixed in 0.01% OsO$_4$ in Kellenberger buffer (Kellenberger et al., 1958). The cell pellet was resuspended in Kellenberger buffer and recentrifuged. Two drops of tryptone salts solution and 2% Nobel agar (Difco) were added after the tube had been placed in a 47°C water bath. The agar-cell suspension was placed on a glass slide, allowed to solidify and cut into small cubes. The sample was post-fixed in 1% OsO$_4$ overnight at room temperature. Staining was carried out in 0.5% uranyl acetate in Kellenberger buffer for 2 h. The samples were dehydrated with increasing concentrations of ethanol and were embedded in Spurr's low viscosity resin (Spurr, 1969). Thin sections were post-stained in 0.5% uranyl acetate in methanol for 15 min to 1 h and then in 0.4% lead citrate for 1 to 5 min (Reynolds, 1963).

For negative stains, the cell samples were added to parlodion-carbon-coated 200-mesh copper grids, and were stained in 1% phosphotungstic acid for 1 min. All preparations were photographed
In a JEOL JEM 100S electron microscope.

A good portion of the electron micrographs were taken by Dr. David Balkwill or Melissa Rochkind of this department.

**Induction and Assay of B-Galactosidase and Tryptophanase**

Cultures were induced for B-galactosidase and tryptophanase by the addition of lactose and tryptophan directly into the fermentation vessel, so the final concentrations in the fermentor vessel was $10^{-3}$ M and $1.5 \times 10^{-3}$ M, respectively. B-galactosidase activity was measured as described by Miller (1972). The amount of enzyme determined in the assay was corrected for culture turbidity and B-galactosidase was reported as the amount of enzyme that will hydrolyze $10^{-9}$ mol (min)$^{-1}$ of ONPG at 20°C. Tryptophanase activity was determined by the method of Bilezikian et al. (1967). Samples of 0.4 ml were delivered into chilled test tubes containing 0.2 ml of toluene, and the tubes were shaken vigorously and kept at 0°C until all samples had been collected. Samples were then vortexed and warmed at 37°C for 15 min. The reaction commenced by the addition of 0.2 ml of a warm solution containing 1.0 mg L-trp and 30 mg of pyridoxal phosphate in 0.12 M Tris-HCl buffer, pH 7.5. The tubes were vortexed and incubated for 1 h before the reaction was stopped by the addition of 1.8 ml of Ehrlich's reagent containing 5 parts of 5% p-dimethyl-amino-benzaledhyde in 95% ethanol and 12 parts of acid alcohol (8 ml conc H$_2$SO$_4$ in 100 ml ethanol). The O.D. was read at 568 nm after 30 min and that value was multiplied by 0.054 to give the μmoles of
Indole formed. An enzyme unit was determined as the amount of enzyme that produces 1 μm mole of indole min⁻¹. Indole in the filtrate was quantified by simply deleting the substrate, L-tryptophan from the reaction mixture and following the same procedure.

**Particle Counts**

Samples drawn from the fermentor were fixed in 0.1% prefiltered formaldehyde and dilutions were counted with a Coulter Counter, model ZB1, fitted with a 30 μm orifice, and connected to a 100 window channelizer with an X-Y recorder. The settings were: manometer, 100 μl; matching switch, 40K; amperature current⁻¹, 0.25; and amperature⁻¹, 0.354.

**Lysogeny of E. coli NF161**

Strain NF161 was lysogenized with λ phage, by growing the organisms aerobically for 18 h in nutrient broth containing ca. 10⁸ λ phage particles per ml. The organism was then subcultured twice on nutrient agar and isolated colonies were selected by their ability to induce λ phage upon exposure to UV light (Witkin, 1976). The phage was quantified by the soft agar overlay procedure. This assay consisted of pipetting 0.1 ml of a virus dilution made in minimal broth on to a petri dish containing nutrient agar and overlaying the virus solution with molten agar containing 0.8% agar and ca. 1 X 10⁸ E. coli CSH22, a lambda-sensitive strain.
Analysis of Filtrate Viral Numbers

The response of a phase 3 culture of NF161-lambda to a pulse of nalidixic acid and to a subsequent pulse of N\textsuperscript{'}-methyl-N\textsuperscript{'}-nitro-N\textsuperscript{'}-nitrosoguanidine was determined by assaying viral particles in the filtrate material with the agar overlay procedure. Nalidixic acid was commonly added directly to the fermentor reaction vessel making the initial concentration in the vessel, 15 \( \mu \text{g ml}^{-1} \).

Starvation of Phase Three Cells

The response of a phase 3 population of \textit{E. coli} to total starvation conditions was determined by shutting off the media input pump and analyzing subsequent, timed samples by plate counting and electron microscopy.

Treatment of Cells with Chloramphenicol

In order to elucidate the number of cells in the D period of the bacterial cell cycle, fermentor samples of strain NF161 were treated with chloramphenicol (Mixakawa et al., 1980). One ml samples were removed from the fermentor and diluted 1:10 in DMM containing: 200 \( \mu \text{g ml}^{-1} \) chloramphenicol; 5 mg ml\textsuperscript{-1} glucose; and 100 \( \mu \text{g ml}^{-1} \) of arginine and methionine. One ml of that dilution was immediately fixed in 1% formaldehyde as the control, and the remaining solution incubated on a rotary shaker at 30\(^\circ\)C for up to 3 h, at which point a 1 ml aliquot was fixed in formaldehyde. All samples were counted in
a coulter counter and the increase in chloramphenicol treated cell numbers in relation to control cell numbers classified as the % of D period cells. Actual particle counts per 100 μl were kept between 10,000 and 30,000 as a negligible coincidence correction occurs within this counting range. A considerable amount of electronic noise or non-bacterial particles were detected in both the control and experimental samples, but no correction was necessary because this background was the same in both samples.
RESULTS

Behavior of *E. coli* B and *E. coli* H10407 In the Recycling Fermentor

Growth of *E. coli* B in the recycling fermentor at a GPR of 390 μ moles h⁻¹ is shown in Figure 6 A and B. Phase one growth shows a lag period followed by exponential growth. When the glucose concentration in the fermentor medium falls below 0.1 μg ml⁻¹, the transport limit of *E. coli* (Chesbro et al., 1979; Shehata and Marr, 1971), phase one ends and phase two begins. In this second phase, the growth rate, dxdt⁻¹, is constant and is directly proportional to GPR. With *E. coli* B, phase two ends at a critical specific growth rate (ca. 0.025 h⁻¹) after 22-24 h have elapsed and phase three commences. The growth rate drops to a new, lower value which then remains constant for at least 150 h. The specific growth rate, μ, falls continuously throughout phase two and phase three, and since mass doubling time in these phases of linear growth is μ⁻¹, the doubling time increases continuously.

Figure 6 A and B also show changes in CFU, biomass, RNA, DNA, and protein before and after phase three cells were subjected to an upshift in GPR. The upshift was imposed by quadrupling the flow rate of medium into the fermentor (FR), increasing the GPR from 390 μ moles h⁻¹ to 1552 μ moles h⁻¹. During phase two, the concentrations of DNA, RNA, and protein increases in constant
Figure 6A (top): Changes in RNA, DNA, and protein during 3 phases and 4X upshift of *E. coli* B.

Changes in RNA ( ), DNA ( ), and protein ( ) concentrations of *E. coli* B grown in a recycling fermentor pH 7.0, 30°C, and at a GPR of 390 μmole h⁻¹. Arrow indicates change in cell parameters when GPR was shifted to 1550 μmole h⁻¹.

Figure 6B (bottom): Changes in mass and CFU during 3 phases and 4X upshift of *E. coli* B.

Changes in CFU ( ) and mass ( ). (Identical growth parameters as in 6 A.)
Figure 6A (top) and Figure 6B (bottom)
proportion. At the onset of phase three, the rates of synthesis of these parameters decrease, but not in proportion to one another and the cells become impoverished in RNA as its synthesis drops by 80% from its phase two rate in comparison.

For the first 6 h after the four-fold upshift, the cell division rate was increased about 13-fold and the rate of biomass increase was raised about 7-fold, greater than their immediately preshift, phase three values. However, the rate of biomass increase was very nearly four times its phase two value. Protein synthesis increased 5-fold and the rate of RNA synthesis about 40-fold. The increase in the rate of DNA synthesis was relatively modest, about 2-fold; however, after 10 h, its rate of synthesis doubled. DNA per unit biomass increased during phase three as RNA per unit biomass decreased (Table 4). Six h post-upshift, these ratios returned to phase two levels. Ten h post-upshift, the rates of RNA synthesis, cell division, and growth commenced falling, apparently toward asymptotes.

Figure 7 shows the growth pattern of _E. coli_ H10407 in the recycling fermentor at a GPR of 412 μmoles h⁻¹. The most striking difference from the pattern observed with the B strain is the length of phase two, here being close to 50 h. When H10407 was grown at differing GPR, the growth rates in phase two and three were dependent on GPR, but the length of phase two remained fixed at 50 h (Elfert, 1979), as it had remained fixed at 22-24 h, despite changes in GPR and growth rate for _E. coli_ B (Chesbro et al., 1979).
TABLE 4. Change in culture parameters when a phase three culture of *Escherichia coli* B was exposed to a four-fold increase in GPR\(^a\)

<table>
<thead>
<tr>
<th>Phase of Growth</th>
<th>DNA/Blomass Ratio(^b)</th>
<th>RNA/Blomass Ratio(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1,2 turnover point</td>
<td>0.022</td>
<td>0.21</td>
</tr>
<tr>
<td>Phase 2,3 turnover point</td>
<td>0.034</td>
<td>0.18</td>
</tr>
<tr>
<td>Phase 3 prior to upshift</td>
<td>0.040</td>
<td>0.10</td>
</tr>
<tr>
<td>Six h post-upshift</td>
<td>0.030</td>
<td>0.17</td>
</tr>
<tr>
<td>Twenty h post-upshift</td>
<td>0.036</td>
<td>0.16</td>
</tr>
</tbody>
</table>

\(^a\)The glucose provision rate was 390 \(\mu\)mole h\(^{-1}\) at an \(F_P\) of 58 ml h\(^{-1}\). At 45 h into phase three, \(F_R\) was increased to 216 ml h\(^{-1}\). Phases are defined in the text.

\(^b\)Ratios are calculated from Figure 6 A and B.
Figure 7: 3 phase growth of *E. coli* H10407.

Growth of *Escherichia coli* H10407 in a recycling fermentor at pH 7.0, 30°C, and a GPR of 412 μmoles glucose h⁻¹.
Accumulation of Cyclic Adenosine 3',5'-Monophosphate

At the time the cells enter phase two, the glucose concentration in the fermentor is lower than 1 ug ml⁻¹ and the PTS system in E. coli is very likely unsaturated (Chesbro et al., 1979). At this phase transition point, and thereafter, β-galactosidase is fully inducible (Evans, 1978). Both conditions indicated the presence of elevated levels of cAMP in the cells, once phase two was entered. Figure 8 shows the total cAMP levels in the culture (both intra and extracellular) throughout the phases. When the cells enter phase two, the basal level of cAMP increases 5-fold and then falls throughout the phase, leveling at about twice the basal level. At the onset of phase three, total cAMP returns to a level similar to that seen at the beginning of phase two and then again falls.

Accumulation of Guanosine Tetraphosphate

The changes in RNA and protein synthetic rates at the entry of the culture into phase three, their relative proportions during phase three, and their changes after the upshift resemble similar changes in many strains of E. coli during carbon-energy shifts (Gallant, 1979; Nierlich, 1978). Such changes have been shown to be closely associated with cellular levels of ppGpp (Gallant, 1979; Nierlich, 1978).

Radioautographs of formic acid extracts of cells from the three phases and after a 4 X upshift in GPR (Figure 13 A) indicated that ppGpp concentrations increased markedly above the basal levels of
Figure 8: cAMP levels in *E. coli* B.

Total levels of $^{32}$P-labelled cAMP (●) in a culture of *E. coli* B grown in a recycling fermentor at pH 7.0, 30°C, and a GPR of 1250 μmoles h$^{-1}$. Growth represented as Klett units (○).
Figure 8
phase one in both phases two and three, and were still above the basal level in the upshifted cells. The chromatograms also revealed the presence of little or no guanosine-5'-triphosphate-3'-diphosphate (pppGpp), the precursor of ppGpp when it is synthesized by the rel A gene product during amino acid shortfall, nor was pppGpp detectable in two-dimensional chromatograms of the extracts. Two-dimensional chromatograms, though, did verify the presence of guanosine tetraphosphate (ppGpp).

Figure 9 shows the concentration of ppGpp in the cell mass at different times in the three phases and after a 4X upshift in GPR. The level of ppGpp in phase two was double its level in phase one and seemed to increase slightly throughout the phase. Its concentration nearly doubled again in phase three cells, then returned close to its early phase two level immediately after the glucose upshift.

**Growth Pattern of RelA, RelX, and SpoT Strains of E. coli In the Recycling Fermentor**

If ppGpp is the determinant of the phase two to phase three transition in the recycling fermentor, mutations in the *relA* and *relX* loci, which produce a decreased ability to accumulate ppGpp, should cause a longer phase two and a delay in the appearance of phase three. Conversely, mutations in the *spoT* locus, which diminish ppGpp degrading capability should bring about a more rapid accumulation of ppGpp, thus, accelerating the appearance of phase three and shortening phase two. Thus, the cellular level of ppGpp is the summation of the activities of these gene products.
Figure 2: ppGpp levels in *E. coli* B.

Guanosine tetra phosphate levels (histograph) in *Escherichia coli* B growing in a recycling fermentor at a GPR of 1250 \( \mu \text{moles glucose h}^{-1} \) until shifted to 4800 \( \mu \text{moles glucose at 45 h.} \)

Growth shown as Klett units (\( O\)), pH 7.0, 30°C.
Figure 9

Klett Units

Hours

Figure 9
*E. coli* strains mutated in the ppGpp regulatory loci were obtained from J. Gallant at the University of Washington, and their growth in the fermentor examined. The pedigree of the strains is shown in Figure 10. The growth patterns of five combinations of these mutants are shown in Figure 11. The mutant loci affected the growth phase pattern in the recycling fermentor by lengthening or shortening phase two in a manner exactly predictable from the role their products play in the synthesis and degradation of ppGpp, thus, the supposition that it is the accumulation of ppGpp that brings about phase three growth. That is, the faster ppGpp accumulates in the cells as the result of the activity of the relA<sup>+</sup>, relX<sup>+</sup>, and relS<sup>+</sup> products, the sooner will the level be reached that initiates phase three by restricting transcription of rRNA.

Strain NF1035 (relA, relX), although it retains some capability for ppGpp synthesis (Pao and Gallant, 1978), has the most severely restricted capability for ppGpp production. This restricted synthesizing ability coupled with its possession of an active ppGppase led to the expectation that it would either have the longest phase two of any strain studied, or would not enter phase three at all. It did enter phase three after the longest phase two yet observed, suggesting that ppGppase activity was ultimately depressed enough by either energy deficiency, or accumulation of uncharged tRNA, or both, to permit an accumulation of ppGpp to the level necessary for phase three growth behavior to begin.
**Figure 10:** Pedigree of *E. coli* rel mutants.

Pedigree of the mutant strains shown in Figure 11.
P1(PA2-31) x NF-53  (select for thyA+argA relA+)

NF-161  (F- metB1 relA+ argA52 spoT1λλ)

NF-254  (metB argA pyrE spoT1 relA+)

NF-859  (metB argA relA+ spoT+)

CP-79  (relA- relX- spoT+)

NF-1035  (relA- relX- spoT+)

NF-859X  (metB argA relA spoT+ fuc-)

1 by NMG mutagenesis and penicillin enrichment

2 P1(NF-809) x NF-254  (select pyrE+ spoT+)

3 transduction of the relA- allele with arg+ from the relA-2 strain

Figure 10
Figure 11: 3 phase growth of five *E. coli* rel mutants.

Growth of a wild type and *rel* system mutants of
*E. coli* in a recycling fermentor at pH 7.0, 30°C.
From top to bottom, the cultures and respective
GPR in μmoles glucose h$^{-1}$, were NF161 (*spoT*),
416, NF162 (rel A1, *spoT*), 408; NF859 (wild type),
433, NF859X (rel A1), 416; and NF1035 (rel A1,
rel X), 421.
Figure 11
Figure 12 shows the changes in RNA synthesis during the growth of strains NF161 and NF1035, which had the longest and shortest phase two growth intervals, respectively. The abrupt decrease observed in RNA synthesis at the phase two/phase three transition was consistent with the expected effect of an increase in ppGpp concentration at that time. The other strains showed similar changes in RNA synthesis.

The effect of the individual loci upon the length of phase two is shown in Table 5. The length of the phase is affected in a remarkably precise way by the alleles of relA+, relX+, and spoT+ genes. A change in the spoT locus has about three times the effect upon the length of phase two that a change in the relX locus has and about four times that of a change in the relA locus. The relA+ \( \rightarrow \) relA change lengthens phase two by 11-12 h; the relX+ \( \rightarrow \) relX change lengthens it by 15 h, and the spoT+ \( \rightarrow \) spoT change shortens it by 44-45 h. However, changes in the loci had no apparent effect of \( Y_a \) in phase two growth, which clustered about an average figure of 0.016 mg cell dry weight \( \mu \)mole glucose\(^{-1}\). There did seem to be a trend in \( Y_a \) values for phase three growth that was inversely correlated with the length of phase two: \( Y_a \) for phase three decreased from 0.007 mg cell dry weight \( \mu \)mole glucose\(^{-1}\) for strains NF161 to 0.005 mg cell dry weight \( \mu \)mole glucose for strain NF1035.

The rate of change of various culture parameters is shown in Table 6. These data indicate that phase two is a period of balanced growth with DNA, RNA, protein, bacterial dry weight and bacterial
Figure 12: Accumulation of RNA in two *E. coli* rel mutants.

Accumulation of RNA by *E. coli* (•) NF161 and
(○) NF1035 in the experiments shown in Figure 11.
Figure 12
TABLE 5. Apparent growth yields, $Y_a$, and lengths of the phase two growth interval for the *Escherichia coli* strains of known *rel* system genotype

<table>
<thead>
<tr>
<th>Strain</th>
<th>$Y_a$ (mg biomass μmole glucose$^{-1}$)</th>
<th>Length of Phase 2 (h)</th>
<th>Length of Phase 3 (h)</th>
<th>Increase or decrease in length (h) of phase 2 associated with mutant allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF161 (spoI)</td>
<td>0.017</td>
<td>13</td>
<td></td>
<td>+11</td>
</tr>
<tr>
<td>NF162 (relA1, spoI)</td>
<td>0.014</td>
<td>24</td>
<td></td>
<td>-44 -45</td>
</tr>
<tr>
<td>NF859 (wild type)</td>
<td>0.016</td>
<td>57</td>
<td></td>
<td>+12</td>
</tr>
<tr>
<td>NF859X (relA1)</td>
<td>0.014</td>
<td>69</td>
<td></td>
<td>+15</td>
</tr>
<tr>
<td>NF1035 (relA2, relX)</td>
<td>0.016</td>
<td>84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 6. Rate of change of parameter expressed as \( \mu g \text{ ml}^{-1} \text{ h}^{-1} \) from experiments in Figure 11.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Phase</th>
<th>Dry Wt</th>
<th>Protein</th>
<th>RNA</th>
<th>DNA</th>
<th>Bacterial Numbers&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF161</td>
<td>2</td>
<td>18.0</td>
<td>11.0</td>
<td>1.2</td>
<td>1.0</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.1</td>
<td>4.0</td>
<td>0.5</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td>NF162</td>
<td>2</td>
<td>14.0</td>
<td>9.0</td>
<td>1.3</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.0</td>
<td>3.1</td>
<td>0.6</td>
<td>ND</td>
<td>0.2</td>
</tr>
<tr>
<td>NF859</td>
<td>2</td>
<td>15.0</td>
<td>8.6</td>
<td>1.6</td>
<td>ND</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.1</td>
<td>3.0</td>
<td>0.6</td>
<td>ND</td>
<td>0.3</td>
</tr>
<tr>
<td>NF859X</td>
<td>2</td>
<td>12.7</td>
<td>ND</td>
<td>1.9</td>
<td>ND</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.0</td>
<td>ND</td>
<td>0.5</td>
<td>ND</td>
<td>0.1</td>
</tr>
<tr>
<td>NF1035</td>
<td>2</td>
<td>14.3</td>
<td>9.0</td>
<td>1.1</td>
<td>0.6</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.0</td>
<td>5.2</td>
<td>0.2</td>
<td>0.1</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as percent increase in parameter per h.

<sup>b</sup>Not determined.
numbers increasing at constant rates with respect to each other, regardless of the length of the phase. As the cells enter phase 3, the rates of macromolecule accumulation differ in respect to each other, reflecting an unbalanced growth state. Bacterial numbers increased exponentially in phase one, then became linear, or approximately so in phase two. At the onset of phase three, the rate of increase fell, but remained above zero in most cases as far as our level of detection could discern.

The factor critical to the phase two/three shift appears to be the specific growth rate, $\mu$. Chesbro et al. (1979) found that the length of phase two is independent of growth rate; changes in the GPR only affected the slope of the line in both phases two and three, and that the two/three turnover point occurred at the same specific growth rate (Chesbro et al., 1979). The phase two, three point, specific growth rates for the rel mutant strains, *E. coli* B and H10407 are listed in Table 7. A particularly striking observation is that *E. coli* B and the H10407 strains possess the same specific growth rate at the phase two/three turnover point as strains NF162 and NF859, respectively. *E. coli* B and the H10407 strain also showed phase two lengths corresponding respectively to the NF162 (relA, spoT) and NF859 (wild type) strains; the same $Y_a$ for phase two as the NF strains; and the same $Y_a$ for phase 3, respectively, as NF162 and NF859 (Figures 6B, 7, 11).
TABLE 7. Specific growth rates (μ) (X⁻¹ dx/dt⁻¹; mg biomass increment mg⁻¹ biomass h⁻¹) at phase two, three turnover point

<table>
<thead>
<tr>
<th>Strain</th>
<th>μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>aE. coll NF161</td>
<td>0.030</td>
</tr>
<tr>
<td>aE. coll NF162</td>
<td>0.022</td>
</tr>
<tr>
<td>aE. coll NF859</td>
<td>0.012</td>
</tr>
<tr>
<td>aE. coll NF859X</td>
<td>0.011</td>
</tr>
<tr>
<td>aE. coll NF1035</td>
<td>0.011</td>
</tr>
<tr>
<td>bE. coll B</td>
<td>0.024</td>
</tr>
<tr>
<td>cE. coll H10407</td>
<td>0.012</td>
</tr>
</tbody>
</table>

a Calculated from Figure 11.
b Calculated from Figure 6 B.
c Calculated from Figure 7.
The RelA Phenotypes of Escherichia coli B and H10407

The absence of pppGpp from radioautograms of *E. coli* B suggested that the *relA* gene product system which synthesizes ppGpp as an intermediate was not active in this strain. A test was made of all the strains studied by the method of Engel et al. (1979) which subjects cells simultaneously to energy and amino acid starvation. The results are shown in Figure 13 B.

A carbon-energy downshift of the sort imposed by the recycling fermentor subjects the cells to both an energy shortage and amino acid starvation as the supply of glucose-derived intermediates per cell dwindles. The resemblance of the growth pattern of *E. coli* B in the fermentor to that of strain NF 162 (*relA1*, *spoT1*), and of strain H10407 to strain NF859 (*relA*+, *relX*+, *spoT*+) led to the expectation that each pair of strains should show corresponding behavior in the accumulation of pppGpp and ppGpp when tested by the procedure of Engel et al. (1979). Figure 13 B shows that *E. coli* H10407 and NF859 produced both nucleotides, whereas *E. coli* B produced only ppGpp as did NF162 and NF859X (*relA1*). Strain NF161 (*relA*+, *spoT*) which is isogenic with NF162, produced a small but detectable amount of pppGpp in addition to ppGpp.

The resemblance of the growth pattern of *E. coli* B to the NF162 (*relA*, *spoT*) strain suggested that it might also be *spoT*-. The stability of ppGpp in *E. coli* B was tested by treating phase three cells with arsenate. Simple downshift in GPR in phase three produced rapid cell death and lysis (Figures 16, 17, 18) which was a
Time sequence of one-dimensional chromatography of $^{32}$P-labeled formic acid extracts from *E. coli* B grown in the recycling fermentor at a GPR of 1250 µ moles h$^{-1}$. GPR was shifted to 4800 µ moles h$^{-1}$ at 45 h.

One-dimensional chromatography of $^{32}$P-labeled formic acid extracts from cells grown according to the procedure of Engel et al. (1979). Nucleotides were identified by internal markers. Strains from left to right are: *E. coli* NF161, NF162, NF859, NF859X, *B. polymyxa* ATCC25901, *S. typhimurium*, *E. coli* H10407, P307, and *E. coli* B.
Figure 13A (top) and Figure 13B (bottom)
complication that was avoided by using arsenate poisoning to curtail the cells' energy supplies. This treatment resulted in no overt lysis during the interval examined. While other polyphosphorylated nucleotides declined rapidly in concentration, e.g. the half life of guanosine triphosphate, shown in Figure 14 for comparison, was on the order of 3 min, ppGpp had a half life (Figure 14) greater than 16 min. Since ppGpp can have a half life or less than one min in spoT+ cells (Fill et al., 1977) either E. coli is also spoT−, or arsenate inhibited the ppGpp pyrophosphatase in some way, perhaps by lowering the transmembrane proton gradient which has been suggested (Tetu et al., 1980) as important to the in vivo activity of the enzyme.

**Cell Size Determination**

The rate of change of cell numbers (Table 8) in relation to bacterial dry weight indicated that changes in cellular geometry were occurring in phases two and three with the E. coli NF strains. Measurements of electron micrographs of negatively stained cells supported this observation (Table 8 and Figure 15). As the cells entered their respective second phase of growth the mean cell length diminished. This was followed by a gradual increase in cell size and volume, which lasted the remainder of the experiment.
Figure 14: Nucleotides in *E. coli* B after arsenate treatment.

Effect of 0.3 g of potassium arsenate on pools of G-4-P (○) and GTP (●) in a recycling fermentor culture of *E. coli* B, detected by autoradiography. GPR is 1250 μmoles h⁻¹, pH 7.0, 30°C.
Figure 15: Cell sizes of \textit{E. coli} rel mutants during 3 phase growth.

Cell size determinations made from negatively stained cells of strains NF859 (\textbullet{}), NF161 (\textcircled{O}), and NF162 (\textblacksquare{}) from experiments shown in Figure 11. Vertical bars represent standard deviations (see Table 8).
Figure 15
Figure 16: Response of cell numbers and culture turbidity to starvation of E. coli B.

Response of cell numbers ( □ ) and culture turbidity ( □ ) to the shutoff of medium addition pump in a phase 3 culture of E. coli B. Starvation started at 49 h. GPR 412 μmoles h⁻¹, pH 7.0, 30°C.
Figure 16

- 29.5% cell death
- 99% cell death

Turbidity (Klett units)

Bacterial Numbers (log CFU/mL)

Post-inoculation Time (h)

20 40 60 80 100 120 140 160 180
Figure 17: Electron micrographs of starved *E. coli* B cells.

Figure 17A (top left): Phase 3 cells of *Escherichia coli* B 4 h after the nutrient flow to the fermentor was stopped. Arrows point to dividing cell showing incipient lysis. Adjacent to it is a typical 3 component cell ghost. Figure 17 B,C (top right and middle, left, respectively): represent cells 4 h after a nutrient shutoff. Arrows point to areas of possible septum disruption. Figure 17 D,E (middle, right, and left, respectively): represent cells 17 h after a nutrient shutoff. Arrows point to areas of septum disruption. Figure 17 F shows phase 3 cells, 4 h after a nutrient shutoff. A 3 component cell ghost is visible in which the cell wall can be seen to have ruptured in the septal area and curled back on itself. Photo credit, D.L. Balkwill.
TABLE 8. Cell measurements of negatively stained cells\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Post-inoculation time</th>
<th># cells measured</th>
<th>mean length ± SD\textsuperscript{b}</th>
<th>mean width ± SD\textsuperscript{b}</th>
<th># dividing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early 1</td>
<td>5</td>
<td>70</td>
<td>2.553 ± 0.605</td>
<td>0.717 ± 0.083</td>
<td>ND</td>
</tr>
<tr>
<td>Late 2</td>
<td>25</td>
<td>130</td>
<td>1.785 ± 0.406</td>
<td>0.717 ± 0.107</td>
<td>ND</td>
</tr>
<tr>
<td>Late 3</td>
<td>47.5</td>
<td>72</td>
<td>2.035 ± 0.425</td>
<td>0.855 ± 0.071</td>
<td>ND</td>
</tr>
<tr>
<td>Early 1</td>
<td>3.5</td>
<td>85</td>
<td>2.357 ± 0.660</td>
<td>0.900 ± 0.092</td>
<td>1</td>
</tr>
<tr>
<td>Late 2</td>
<td>23</td>
<td>129</td>
<td>1.988 ± 0.486</td>
<td>0.880 ± 0.104</td>
<td>8</td>
</tr>
<tr>
<td>Late 3</td>
<td>59.5</td>
<td>159</td>
<td>2.315 ± 0.664</td>
<td>0.787 ± 0.087</td>
<td>10</td>
</tr>
<tr>
<td>Early 1</td>
<td>5</td>
<td>39</td>
<td>4.125 ± 2.360</td>
<td>0.882 ± 0.126</td>
<td>6</td>
</tr>
<tr>
<td>Mid 1</td>
<td>7</td>
<td>62</td>
<td>2.419 ± 0.789</td>
<td>0.794 ± 0.113</td>
<td>10</td>
</tr>
<tr>
<td>Late 1</td>
<td>10.5</td>
<td>117</td>
<td>2.499 ± 0.601</td>
<td>0.813 ± 0.090</td>
<td>1</td>
</tr>
<tr>
<td>Late 2</td>
<td>54</td>
<td>90</td>
<td>1.776 ± 0.485</td>
<td>0.916 ± 0.142</td>
<td>5</td>
</tr>
<tr>
<td>Late 3</td>
<td>99.5</td>
<td>121</td>
<td>1.895 ± 0.555</td>
<td>0.942 ± 0.112</td>
<td>7</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Determined from experiments in Figure 11.

\textsuperscript{b} Measurements expressed in \textmu m; SD = standard deviation of replications.
Figure 18: Electron micrographs of starved and upshifted *E. coli* B cells.

Figure 18 A (top, left): Phase 3 cells of *E. coli* B 4 h after a nutrient shutoff. Arrows point to area of sepal disruption. Figure 18 B (top, right) shows phase 3 cells 17 h after a nutrient shutoff. Arrows point to another less common area of cell disruption.

Figure 18, C, D (middle, right, and left, respectively), shows phase 3 cells 4 h after a four-fold nutrient upshift of the starved population. Three component cell ghosts are present, with arrows at areas likely to be the points of septum disruption.

Figure 18 E (bottom, left) shows phase 3 cells, 4 h after a four-fold nutrient upshift. Arrows point to dividing cells either intact, or partially lysed, illustrating the assymetry of division in phase 3 cells. Figure 18 F (bottom, right) shows phase 3 cells of *E. coli* B after a four-fold nutrient upshift. Arrows point to areas of incipient septum formation. All growth parameters the same as Figure 16. Photo credit, D.L. Balkwill.
Glucose Downshifts

When *E. coli* is subjected to a glucose downshift in phase three by changing the GPR to zero, 30% of the population dies within three h after the downshift. This is followed by a period of little or no further death which lasts for approximately 24 h. At the termination of this passive period, death begins again at a rapid rate, so that 90% of the population is dead after another 15 h (Figure 16). In a repetition of the experiment in Figure 16, the behavior of phase three cells following a nutrient downshift was studied by thin section, transmission electron microscopy. A characteristic pattern of disruption was observed. Figures 17 A, B, C, D, E, F, and 18 A, C, and D all disclose cells that have been disrupted or "blown-out" at an area of incipient septation.

When these energy starved cells were exposed to a four-fold energy upshift by quadrupling the flow rate into the reaction vessel, thin sections by electron microscopy revealed that the now rapidly dividing cells showed signs of asymmetric division (Figure 18 E). Doubly dividing cells also became evident (Figure 18 F), suggesting that it is cells dividing in this manner which gave rise to the tripartite ghosts.

Reycling Fermentor Growth of *Bacillus polymyxa*

The stringent response seems to be ubiquitous to prokaryotes (Silverman and Atherly, 1979) and thus, it was anticipated that the growth pattern behavior of *E. coli* in the recycling fermentor may be
more generally applicable to bacterial genera. To examine this hypothesis, the organism, *Bacillus polymyxa*, was studied. To avoid complications with sporulation, an asporogenous mutant was used.

The growth patterns observed for *B. polymyxa* at three pH values (Figure 19 A, B, C) correspond closely to that observed for *E. coli* at pH 7.0 (Chesbro et al., 1979). There were three successive growth phases. The first phase was essentially batch-type growth which reaches exponential rates. When the glucose initially in the growth vessel was exhausted and becomes effectively zero, the growth rate became linear and dependent on the GPR. Phase two terminated after 30 h, and phase three began and maintained itself for an indefinitely determined length of time.

Consequently, the descriptions of the growth of *B. polymyxa* in phases two and three were the same as those applying to *E. coli* (Chesbro et al., 1979; Arbige and Chesbro, 1982). Where the growth rate is constant within a respective phase, the specific growth rate, $\mu$, decreases continuously within phases two and three, with an abrupt decline in its own rate of change in the transition between phases. Additionally, the mass doubling time which is the reciprocal of $\mu$, increases throughout both phases two and three.

Table 9 shows the tabulations of growth rate, during phases two and three and for the first 5 h following a four-fold upshift in GPR made in phase three during the experiments shown in Figure 1. Values of $\mu$ and the mass doubling time at the start and end of phase two, the start of phase three, and immediately before and after the
Figure 12: 3 phase growth of *Bacillus polymyxa*.

Growth of *Bacillus polymyxa* in a recycling fermentor at: (A) pH 5.8; (b) pH 6.2; (C) pH 7.0. The glucose provision rate (GPR) was 490 μmoles h⁻¹ for all three runs. The first growth phase extended from 0 h to the first arrow, the second growth phase extended from the first to the second arrow, and the third growth phase from the second to the third arrow. At the time corresponding to the third arrow, the GPR was increased four-fold to 1960 μmoles h⁻¹.
Figure 19
TABLE 9. Growth rates, specific growth rates, and mass doubling times of *Bacillus polymyxa* growing at 3 pH values at 30°C in a recycling fermentor with energy limiting.

<table>
<thead>
<tr>
<th>Phase</th>
<th>pH</th>
<th>Growth rate ((dX/dt; \text{mg biomass h}^{-1}))</th>
<th>Specific growth rate ((X^{-1}dX/dt; \text{mg biomass increment mg}^{-1} \text{ biomass h}^{-1}))</th>
<th>Mass Doubling Time ((h))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>start</td>
<td>end</td>
<td>start</td>
</tr>
<tr>
<td>2</td>
<td>5.8</td>
<td>6.75</td>
<td>6.0 (\times) 10^{-2}</td>
<td>2.0 (\times) 10^{-2}</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>8.24</td>
<td>6.1 (\times) 10^{-2}</td>
<td>2.3 (\times) 10^{-2}</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>5.99</td>
<td>4.8 (\times) 10^{-2}</td>
<td>2.0 (\times) 10^{-2}</td>
</tr>
<tr>
<td>3</td>
<td>5.8</td>
<td>2.75</td>
<td>8.4 (\times) 10^{-3}</td>
<td>6.4 (\times) 10^{-3}</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>3.60</td>
<td>1.1 (\times) 10^{-2}</td>
<td>7.8 (\times) 10^{-3}</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>3.29</td>
<td>1.1 (\times) 10^{-2}</td>
<td>7.9 (\times) 10^{-3}</td>
</tr>
<tr>
<td>Fourfold upshift^a</td>
<td>5.8</td>
<td>20.7</td>
<td>4.8 (\times) 10^{-2}</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>30.5</td>
<td>6.6 (\times) 10^{-2}</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>38.3</td>
<td>9.7 (\times) 10^{-2}</td>
<td>---</td>
</tr>
</tbody>
</table>

^aThe fourfold upshift was imposed in phase 3, (the values for "end" in phase 3 refer to the period immediately before the upshift), by increasing \(F_p\) to the fermentor fourfold, changing the glucose provision rate from 490 \(\mu\)mol h\(^{-1}\) to 1960 \(\mu\)mol h\(^{-1}\).
four-fold upshift in phase three are also tabulated.

The growth rate immediately after upshift varied from 3.0 to 6.4 times the growth rate in phase two. Post-upshift values of $\mu$ and the mass doubling time were very close to their values at the commencement of phase two. Thus, the cells returned to and expressed the potential for growth possessed in phase two, 65 h previous to the upshift when energy first became limiting, rather than that exhibited immediately prior to the upshift in phase three. Figure 20 shows concentrations of DNA, RNA, and protein in the fermentor during the course of the experiment at pH 6.2 in Figure 19. Accumulation of these macromolecules remained balanced in their proportions during phase two, but upon entry into phase three, the rate of RNA synthesis slowed more than that of the other species. During the first few hours of the upshift, the rate of RNA synthesis exceeded that of protein and DNA synthesis.

To examine the ability of this strain of *B. polymyxa* to synthesize the regulatory guanine nucleotides, it was tested by the procedure of Engel et al. (1979). Figure 13 B shows that *B. polymyxa* formed both pppGpp and ppGpp, which corresponds to the behavior of relA$^+$ *E. coli* strains.

**Fermentation Behavior of *B. polymyxa* In the Recycling Fermentor**

The three pH realms of growth of *B. polymyxa* (Figure 19) were chosen in an attempt to reveal the effect of pH *per se* upon behavior in the recycling fermentor while the path of dissimilation
Figure 20: Changes in levels of cellular RNA, DNA, and protein in *B. polymyxa*.

Changes in the biomass levels of RNA (□), DNA (▲), and protein (●) during run B shown in Figure 19.
Figure 20
remained constant during an experiment. A shift in dissimilatory metabolisms as expected to occur at the upper pH, since at the two lower pH values (Wood, 1961) the mass transformation of glucose of *B. polymyxa* in anaerobic batch culture observed by Adams and Stanier (1945) was expected as follows:

\[
1 \text{ mol glucose} \rightarrow 0.67 \text{ mol 2,3 butylene glycol} + 0.67 \text{ mol ethanol} + 2.0 \text{ mol CO}_2 + 0.61 \text{ mol H}_2
\]

(2)

Anaerobic batch culture of the strain of *B. polymyxa* we investigated yielded 84% of the 2,3 butylene glycol expected from the above mass transformation.

2,3 butylene glycol, however, was not detected at any pH realm in the recycling fermentor. Its presence was detectable only early in phase one, due to its presence in the inoculum. The compound was then undetectable, due to washout by the start of phase two. In phases two and three, only ethanol and acetate were detected as non-gaseous products. Their ratios corresponded to those expected from the transformation:

\[
1 \text{ mol glucose} \rightarrow 1 \text{ mol ethanol} + 1 \text{ mol acetate} + 2 \text{ mol CO}_2 + 2 \text{ mol H}_2
\]

(3)

Production of 2,3 butylene glycol by *B. polymyxa* has been reported to be favored by an increased H\textsubscript{2} pressure (Mickelson and Werkman, 1938). Because of the continuous mixing, recycling, and constant N\textsubscript{2} sweep of the culture, this increase could not occur in the recycling fermentor. Increased H\textsubscript{2} pressure is apparently a more
Important factor than pH in influencing the particular route of catabolism. The concentrations of acetate and ethanol in the filtrate (which corresponds to their momentary concentrations in the fermentor) of the experiment at pH 6.2 (Figure 19 B) are shown in Figure 21. When their concentrations stabilized in phase two, they remained at that level throughout phases two and three and throughout the upshift in phase three brought about by quadrupling the flow rate. Thus, the recycling fermentor and the chemostat are alike in that once a steady state concentration of excreted cell product has been reached in the reaction chamber, that level will be independent of the medium input rate (FR) (Chesbro et al., 1979). A change in the concentration of the cell product represents a change in the concentration of the energy substrate in the input medium, $S_R$, or a change in the rate of production by the cells.

At the pre-upshift GPR of 490 $\mu$mol glucose h$^{-1}$, the rates of ethanol and acetate production were each 420 $\mu$mol h$^{-1}$, representing 86% dissimilation of the influent glucose. The remaining 14% of the influent glucose would allow the synthesis of 11.5 mg of cell mass h$^{-1}$, on a carbon transfer basis, for the observed cell composition of 43% carbon (CHO analysis). The presence of amino acids in the medium though, would allow for a greater rate of biomass synthesis. Throughout phase two of the experiment at pH 6.2 (Figure 19 B), the rate of biomass synthesis was 8.2 mg h$^{-1}$, and in phase three, it was 3.6 mg h$^{-1}$. 
Figure 21: Filtrate levels of ethanol and acetate produced by \textit{B. polymyxa}.

Filtrate levels of ethanol (●) and acetate (☐) during run B shown in Figure 19.
Figure 21
Energy Costs of Biosynthesis, Maintenance, and the Stringent Response

During both phases, protein, RNA, and DNA components were present in the filtrate material (Figure 22), as had been found previously for E. coli B (Chesbro et al., 1979). The kinetics with which these various materials appeared differed between them. The level of diphenylamine reactive materials, presumed to be DNA components, remained essentially constant throughout the latter two phases and the upshift, while the levels of orcinol positive material, presumed RNA components and Lowry positive material, presumed proteins, mimicked concurrent changes in the level of biomass in the fermentor.

No accumulation of abnormal or disrupted cells in the fermentor was evident in electron micrographs throughout the experiment, although some fragments and disrupted cells were seen in all three phases. At the comparison rates of accumulation of biomass and filtrate products, half or more of the cells in the fermentor would have had to be dead by phase three to implicate the filtrate material as being of autolytic origin.

Table 10 summarizes the observations on filtrate biosynthetic products and biomass during the experiment at pH 6.2 (Figure 19 B) at two selected points before and after the phase transition between phases two and three. The total biosynthetic mass produced per h (filtrate products plus biomass) at these points in phases two and three were respectively, 12.6 and 9.4 mg. The first figure is 10% above, and the second 18% below the theoretical amount permitted by
Figure 22: Filtrate levels of RNA, DNA, and protein in *B. polymyxa*.

Filtrate levels of RNA (■), DNA (△), and protein (●) during run B shown in Figure 19.
TABLE 10. ATP expenditures by Bacillus polymyxa growing anaerobically in a recycling fermentor with energy limiting at pH 6.2, 30°C.

<table>
<thead>
<tr>
<th>Phases</th>
<th>Y</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
<th>Filtrate yields (mg h(^{-1})) at 40 h (phase 2) and 60 h (phase 3)</th>
<th>Biomass yield (mg h(^{-1})) at 40 h (phase 2) and 60 h (phase 3)</th>
<th>Calculated ATP(^a) requirement for synthesis ((\mu)mol h(^{-1}))</th>
<th>% Available ATP used for bio-filtrate bio-maintenance products</th>
<th>% Available ATP used for bio-maintenance products</th>
<th>% Available ATP used for bio-maintenance products</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.017</td>
<td>0.48</td>
<td>2.0</td>
<td>1.8</td>
<td>8.3</td>
<td>104</td>
<td>261</td>
<td>8</td>
<td>21</td>
<td>71</td>
</tr>
<tr>
<td>3</td>
<td>0.007</td>
<td>0.48</td>
<td>2.9</td>
<td>2.6</td>
<td>3.4</td>
<td>143</td>
<td>107</td>
<td>11</td>
<td>9</td>
<td>80</td>
</tr>
</tbody>
</table>

\(^a\)The GPR was 490 \(\mu\)mol glucose h\(^{-1}\). Assuming that filtrate and biomass (44% carbon) carbon derived from glucose, there were 421 \(\mu\)mol h\(^{-1}\) dissimilated, providing 1.26 millimol h\(^{-1}\) of ATP from the pathway of equation (3). The ATP required for synthesis of filtrate biosynthetic products is calculated from the theoretical yields given by Stouthamer (1979). That is, treating the DNA and RNA as breakdown products, the costs in mol ATP \(\mu\)g\(^{-1}\) polymer for a bacterium growing in a glucose plus amino acids medium is 1.79 \(\times\) 10\(^{-8}\), 1153 \(\times\) 10\(^{-8}\), and 0.36 \(\times\) 10\(^{-8}\) for DNA, RNA, and protein respectively. If the filtrate DNA and RNA are treated as monomeric precursors, rather than breakdown products, the % ATP used for biomass (growth) will be decreased 1% and the % used for maintenance increased 1%. For biomass yield, \(\gamma_{\text{ATP}}\) was taken as 31.9 (Stouthamer, 1979).
carbon transfer from glucose, probably within the error limits of the calculations.

The rate of production of filtrate biosynthetic products in phase three counter balances the drop in biomass synthetic rate (Table 10). This shift from accumulation of biosynthetic materials in biomass to their release into the filtrate, whether due to increased secretion, sloughing or autolysis, accompanies the determinant for phase three, presumably the stringent response (Arbige and Chesbro, 1982). When all the biosynthetic products, that in the filtrate and in the biomass, are accounted for, the change in the amount of available energy used for biosynthesis is 9% ingoing from phase two to phase three. In other words, 9% of the available energy is diverted to maintenance from biosynthesis at the phase three transition.

Behavior of Yersinia pestis in the Recycling Fermentor

To test the sensitivity of Y. pestis to stringent control, the organism was cultured in the recycling fermentor at 26°C in the absence of calcium. Figure 23 shows the growth pattern observed. There was an absence of an exponential growth period (phase one), and growth only became apparent when the N₂ flow to the fermentor was stopped at 15 h. Glucose in the filtrate fell below detectable levels (0.1 μg ml⁻¹) 25 h into the experiment, which is the same level seen at the phase one, two turnover point in both E. coli and B. polymyxa. The growth rate then became linear for 20 h, at which
Figure 23: 3 Phase growth of *Yersinia pestis*.

Growth of *Yersinia pestis* CV 76 in the recycling fermentor, with a GPR of 408 μmoles h⁻¹, 26°C, and pH 7.0. Klett units (□) and RNA biomass (▲).
Figure 24: Phase contrast pictures of *E. coli* NF161 before and after nalidixate treatment.

Figure 24 A (top): Phase contrast picture of *E. coli* NF161 grown aerobically in minimal medium;
Figure 24 B (bottom): represents the same culture after treatment with 20 μg ml⁻¹ nalidixic acid for 3 h.
point the rate slowed and became linear again at a lower level for the remainder of the experiment. RNA accumulated at a steady rate throughout the first period of linear growth, and its rate of accumulation fell sharply at the transition point.

**Induction and Persistence of Enzymes and SOS Activity in the Recycling Fermentor**

The pattern of induction and activity of tryptophanase and B-galactosidase in *E. coli* NF161 (spoT), is shown in Figure 25. Induction and loss of B-galactosidase in this phase two culture was determined by exposing the culture to lactose, a metabolizable inducer, at a concentration of $10^{-3}$ M. Induction and decay patterns were very similar to those of *E. coli* B observed by Evans (1978). Levels of the enzyme peaked, and then rapidly decayed over the next seven h until 50% of the activity disappeared. The rate of decay then slowed, as over the next 20 h only 25% of the remaining activity disappeared. No B-galactosidase was present in the filtrate material, ruling out sloughing and/or autolysis as the cause of enzyme loss. Tryptophanase was induced by the addition of $1.5 \times 10^{-3}$ M L-tryptophan to the culture. Its pattern of induction and activity (Figure 25) was very different than that of B-galactosidase. Cell bound tryptophanase rapidly reached an initial peak, then became linear or approximately so for 20 h before falling. Throughout the initial stages of induction, the cell actively secretes indole which reaches its peak 7 h after it begins. The cell continues to secrete indole at a low level after this peak, as the rate of loss from the
Figure 25. Tryptophanase and B-galactosidase levels in E. coli NF161 before and after a nalidixate treatment in phase 3.

Growth of *Escherichia coli* NF161 (spoT1) in the recycling fermentor before and after treatment with nalidixic acid in phase three. The culture was induced for both B-galactosidase and tryptophanase by the addition of lactose and tryptophan: (□□□), Klett units; (■■■), B-galactosidase activity; (●●●●), tryptophanase activity; (●●●) filtrate indole; GPR = 412 μmoles h⁻¹ @ pH 7.0 and 30°C.
Figure 25
fermentor was slower than what would be expected from simple washout. Secretion of tryptophanase was ruled out, as the filtrate material was analyzed before and after passing it through a 100,000 MW filter. The same level of indole was detected in both instances. Subsequent analysis of the filtrate material for tryptophanase activity proved negative as the removal of the substrate, L-tryptophan, from the reaction mixture had no effect on the levels of indole detected in the filtrate, but reduced the levels detected in the cells by 100%.

Following a pulse of nalidixic acid, a series of changes in the bacterial population ensues. After a very short period of positive growth, biomass accumulation is arrested for 14-16 h. Both cell bound tryptophanase and β-galactosidase activity showed decay and an increased rate of decay, respectively, following the nalidixate pulse, although at different times. Approximately 25% of the tryptophanase activity and 65% of the remaining β-galactosidase activity disappeared over the 14-16 h arrest period (Figure 25). Cell numbers immediately increased by 31% after the nalidixate pulse (Figure 26), and then fell to a level seen before the pulse. When the level of nalidixic acid in the fermentation vessel fell below 1 μg ml⁻¹ through dilution, the rate of biomass accumulation became positive again, although at a rate which was 30% lower than that seen in phase three before the pulse.

An attempt to identify the role of recA protein in the changes in the culture parameters seen in Figures 25 and 26, met with a series of technical difficulties. The "SOS" system and the recA
Figure 26: Changes in CFU in *E. coli* NF161 after a nalidixate treatment in phase 3.

Response of cell numbers (▲) to a pulse of nalidixic acid in strain NF161. Some parameters as in Figure 25.
Figure 26

KLEIT UNITS

HOURS

nal

CFU x 10^7

-112-
protein are induced by agents which cause damage to DNA large enough to produce post-replication gaps. The damage provides the signal for recA, whose subsequent activities in repairing the damage can fix the DNA lesion in the chromosome as a mutation. The experimental strategy was then to add a reversible strand damaging agent, nalidixate, to phase three cells in the fermentor and, then, to periodically remove cells from the fermentor to measure reversion of amino acid auxotrophs or the presence of recA protein itself by autoradiography.

However, once removed from the reaction vessel, the cells immediately start to die from energy starvation, unless supplied with an energy source (Figures 16, 17). On the other hand, addition of an energy source makes it impossible to determine whether "SOS" activity appeared in the energy limited, slow growing cells, which are in the fermentor prior to sampling, or appeared after sampling when energy is added. Figure 27 shows the growth pattern of E. coli AB1157 before and after a nalidixate treatment. Auxotrophic reversions of histidine and arginine amino acid markers were found after the nalidixate pulse, but for the reasons stated above it could not be determined whether reversion occurred in the fermentor, or during plating when all the restrictions of a phase 3 culture were removed. Furthermore, direct measurement of recA protein through autoradiography was not feasible, as batch culture experiments showed that the fermentor would have to be treated with impractically high levels of a radioisotope (\(^{35}\)S) to guarantee its autoradiographic detection.
Figure 27: 3 Phase growth of *E. coli* AB1157
before and after nalidixate treatment.

Growth of *Escherichia coli* AB1157 in the recycling fermentor before and after treatment with nalidixate acid in phase 3. GPR was 410 μmoles h⁻¹, pH 7.0, 30°C.
Figure 27

AB1157

15ug ml⁻¹ nalidixate

KLETT UNITS

HOURS

Figure 27
Cell elongation (see Figure 24B), a trait of SOS induced cells, was also not apparent in AB1157 (Figure 27) after nalidixate treatment, but the level of incoming glucose would have only allowed cell size increases of 3.5% over the period studied. This small increase is probably below the detectable limits of the coulter channelizer system.

A practical way of examining the activity of recA in the fermentor is one which does not require removal of the bacterial cells from the fermentor. Since active recA cleaves a repressor controlling lambda phage production, an indirect measurement is an assay which measures its proteolytic activity. E. coli ATCC33311, a strain carrying a defective lambda phage in which the lac Z gene, responsible for B-galactosidase production, is fused to the left promoter of the phage and under control of the repressor, was obtained and cultured in the recycling fermentor. Figure 28 shows aspects of its properties before and after a nalidixate pulse. The background level of B-galactosidase was increased by 31% following the pulse, concommitant with a 10% decline in cell bound tryptophanase activity. As in the case of NF161 (Figure 25) and AB1157 (Figure 27), mass accumulation in this strain stopped for 14-16 h, beginning again when the level of nalidixic acid in the vessel fell below 1 μg ml⁻¹, but at a rate which was 20% lower than that seen in phase three before the pulse. The initial rapid rate increase of B-galactosidase synthesis, after the pulse, lasted only 4-6 h, and then fell but remained positive for sometime, as its
Figure 28: Growth pattern and enzyme levels of *E. coli* ATCC33311 before and after nalidixate treatment.

Growth of *E. coli* ATCC33311 in the recycling fermentor at 30°C, pH 7.0, and a GPR of 412 µmol h⁻¹ before and after treatment with nalidixic acid. Cells were pulsed with tryptophan where indicated. (□-□), Klett units; (○-○), tryptophanase; (●-●), filtrate indole; (●-●-●), B-galactosidase activity.
Figure 28
specific activity increased by 33% over the latter 32 h. After the initial decline, the cell bound tryptophanase remained relatively stable, as its level changed very little over the latter 40 h of the experiment.

Figure 29 shows the results of E. coli strain NF161-lambda (lysogenized with lambda phage) before and after a phase three culture was pulsed with nalidixate. Free lambda phage in the bacterial inoculum used to seed the fermentor appeared to adsorb rapidly to the growing microbial population in the fermentor. The number of detectable filtrate viruses dropped by $7.6 \times 10^4$ ml$^{-1}$ between 5 and 8 h, while colony forming units increased by $3.3 \times 10^8$ ml$^{-1}$. During this period washout of the virus accounted for the loss of $5.0 \times 10^4$ virus ml$^{-1}$, subsequently allowing the adsorption of approximately one virus particle for every $10^4$ microbial cells. Filtrate viral numbers leveled near zero at approximately 19 h, and remained so until the culture was treated with nalidixate. Following the pulse, colony forming units fell until 38% of the population died. Filtrate virus levels jumped ten-fold over the basal level, but assuming that adsorption occurred here also, this level is probably higher. When the concentration of nalidixic acid fell below 1 µg ml$^{-1}$, mass accumulation began, virus levels approached zero, and an increase in colony forming units was detected. Following exposure to a large dose of the powerful methylating agent, MMNG, another burst of phage was apparent and better than 95% of the cells were rendered non-viable.
Figure 29: Viral numbers in *E. coli* NF161 λ before and after nalidixate treatment.

Three phase growth of *E. coli* NF161 in the recycling fermentor before and after a pulse of nalidixic acid, followed by a subsequent pulse of MMNG. GPR was 412 μmoles h⁻¹ at 30°C and pH 7.0. (□), Klett units; (▲), free phage (λ).
Figure 29

Graph showing the effects of various concentrations of antimicrobials on bacterial growth and plaque formation. The x-axis represents time in hours, and the y-axis represents Klett Units. Key points include:

- 1.4 x 10^9 cfu
- 1.3 x 10^9 cfu
- 8.0 x 10^8 cfu
- MMNG, 20 ug/ml
- 15 ug/ml
- 4.0 x 10^8 cfu

The graph illustrates the decrease in bacterial growth and plaque formation over time with the addition of various concentrations of antimicrobials.
Response of Cells to Chloramphenicol

It has become apparent that the slow growing microbial cells produced in the recycling fermentor must remain in various stages of the cell cycle for extremely long periods of time. To test this hypothesis, and to establish what percent of the cells are in the D period of the cycle, cells were treated with chloramphenicol. Chloramphenicol prevents the protein synthesis which is necessary for the initiation of septum formation, and would therefore, prevent any cells in the C period from laying down a septum and undergoing division. D period cell division, however, would be resistant to chloramphenicol. Table 11 summarizes the results obtained when samples of *E. coli* NF161 (Figure 30) were removed from the fermentor and treated with chloramphenicol. During phases one and two approximately 10% of the microbial population appeared to be in the process of septum formation. Early in phase three, this level increased to approximately 30% and remained relatively close to that level for the remainder of the experiment.

Figure 30 also shows the outcome when a phase three culture of NF161 is induced for tryptophanase. The pattern and levels of induction are similar to phase two induced cultures (Figure 25). Following a nalidixate pulse, 13% of the cell bound tryptophanase activity is destroyed. Viable cell counts increase following the pulse by 19% and then quickly fall to a level seen below that prior to the pulse.
TABLE 11. Percent Increase in particle counts when fermentor samples of NF161 were treated with chloramphenicol (D period cells)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Hours post seeding</th>
<th>% D period cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1</td>
<td>5.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Mid phase 2</td>
<td>12.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Late phase 2</td>
<td>15.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Early phase 3</td>
<td>28.5</td>
<td>30.0</td>
</tr>
<tr>
<td>Mid phase 3</td>
<td>30.5</td>
<td>28.5</td>
</tr>
<tr>
<td>Late phase 3</td>
<td>41.0</td>
<td>20.2</td>
</tr>
<tr>
<td>Late phase 3</td>
<td>46.0</td>
<td>35.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Same growth conditions as in Figure 30.
Figure 30: Tryptophanase levels in *E. coli* NF161 before and after nalidixate treatment.

Growth of NF161 in the recycling fermentor before and after a pulse of nalidixic acid, when a phase 3 culture was pulsed with tryptophan. Some parameters as in Figure 25. ( ), Klett units; ( ), filtrate indole; ( ), cell bound tryptophanase.
Figure 30

15 μg ml⁻¹ nalidixate

KLETT UNITS

INDOLE (nmoles)

Figure 30
TABLE 12. Apparent growth yields, $Y_a$, for *Yersinia pestis* and *Escherichia coli* strains ATCC33311 and NF161 (before and after phase three cultures were pulsed with 15 μg nalidixate)

<table>
<thead>
<tr>
<th>Strain</th>
<th>$Y_a$, mg biomass μmol glucose$^{-1}$</th>
<th>phase 2</th>
<th>phase 3</th>
<th>when growth resumes after nalidixate pulse</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. pestis</em></td>
<td></td>
<td>0.019</td>
<td>0.008</td>
<td>-</td>
</tr>
<tr>
<td>NF161$^b$</td>
<td></td>
<td>0.018</td>
<td>0.006</td>
<td>0.004</td>
</tr>
<tr>
<td>ATCC33311$^c$</td>
<td></td>
<td>0.019</td>
<td>0.007</td>
<td>0.005</td>
</tr>
</tbody>
</table>

$^a$Determined from Figure 23.

$^b$Determined from Figure 25.

$^c$Determined from Figure 28.
DISCUSSION

A continuous culture apparatus, with a 100% biomass recycle, constructed by Chesbro et al. (1979) was used to study slow growth of microorganisms. Growth patterns in the recycling fermentor could not be described by simple mass transfer analysis as predicted by Pirt's (1965) mathematical treatment of the current maintenance energy theory. In all organisms studied, the growth rate never approached zero, as some of the available energy was always partitioned to biomass fabricating processes. An apparent flaw in Pirt's mathematical analysis of the maintenance energy concept (equation 1) then seems to be the inherent assumption that the rates of biosynthetic and metabolic reactions would remain constant during energy shortfall. This assumption is not valid when cells are cultured in the recycling fermentor. As growth proceeds in the recycling fermentor, the energy level per cell drops, which in turn triggers the synthesis of the powerful regulatory nucleotides, guanosine tetraphosphate and guanosine pentaphosphate (Figure 9 and Figure 11), the mediators of the bacterial stringent response (Gallant, 1979). In phase three, the ultimate growth stage we have been able to produce in the fermentor, the cellular level of ppGpp (Figure 9), is comparable to levels produced in populations of E. coli by carbon energy source downshifts (Johnson et al., 1977; Pao and Gallant, 1978; Winslow, 1971), and during this period the rates
of biosynthesis of cell macromolecules, i.e. DNA, RNA and protein, are differentially altered (Figure 6 A and Table 4). A number of other biosynthetic reactions, such as the synthesis of bacterial hull components, are restricted during the stringent response (Cashel, 1975) and would certainly affect the reasoning behind Pirt's mathematical deductions of energy limitation.

Energy deficient cells have been produced as a means of studying ppGpp synthesis and its effects on the regulation and coordination of macromolecular synthesis, by a variety of ways. These strategies include cells being shifted from a more preferred substrate, usually glucose, to a less preferred one, such as succinate (Lazzarini et al., 1971); permitting energy substrate exhaustion to occur (Bouquet et al., 1973; Jacobson and Jacobson, 1980); starvation by temperature shifts that inhibit thermally sensitive steps of energy pathways (Cashel, 1975; Lund and Kjeldgaard, 1972); exposure of anaerobes to oxygen (Glass et al., 1978), or imposition of anaerobiosis on aerobic cultures (Lund and Kjeldgaard, 1972); use of analogs competitive with energy substrates (Hansen et al., 1975; Johnsen et al., 1977); and employment of drugs that uncouple oxidative phosphorylation or inhibit substrate level phosphorylation (Cashel, 1975; Richter, 1980 b). It has been argued (Jacobson and Jacobson, 1980) that categorizing all these strategies as "energy-source shiftdown" is questionable since the different treatments affect regulatory controls in exceedingly different ways and accompanying information about their effects on intermediary metabolism and cellular energy
balance is usually absent.

Some of these criticisms are avoided in the production of energy deficient cells in the recycling fermentor, since dissimilatory metabolism as reflected in fermentation balances is constant throughout the three phases (Figure 22), biomass yield per unit glucose utilized is constant within each phase (Figures 6, 7, 11, 19), and the composition of the milieu is constant throughout the phases unless experimentally altered (Chesbro et al., 1979).

A significant characteristic of the recycling fermentor as a scheme for turning on processes that regulate and coordinate biosynthesis at the genetic and metabolic level during energy shortfall is the expansion of the periods of time over which these processes are evoked: the increase in time between basal and maximum observed levels of ppGpp in cells growing exponentially with doubling times of 2-3 h is less than 1 h, while the comparable time for *E. coli* B is the length of phase 2, i.e. 22-24 h (Figure 9).

The length of phase two is constant for a given strain of *E. coli* and independent of growth rate (Chesbro et al., 1979). Its length is apparently determined by a cellular "clockwork". The termination of phase two coincides with the accumulation of a ppGpp level sufficient to prevent RNA accumulation (Figure 6 B, 12). The "clockwork" that determines the length of phase two is the linkage of ppGpp synthesis and degradation to the rate of energy supply per cell.

It was not surprising to encounter elevated levels of ppGpp in
phase three cells because the rates of macromolecular synthesis in this phase resemble in their relative proportions those in cultures undergoing the stringent response. However, the elevated and rising levels of ppGpp in phase two cells, which in the constant proportions of their macromolecular synthesis, resemble cells in steady state growth was unexpected. This intermediate level of ppGpp, although not perceptively diminishing the relative rate of RNA synthesis in phase two cells, is apt to be important to the previously observed slowing of cell division rate in phase two accompanied by increasing cell diameters (Chesbro et al., 1979). Since ppGpp is known to affect phospholipid and peptidoglycan synthesis, and thus, membrane synthesis and cell elongation at the enzyme action level (Gallant, 1979; Nierlich, 1978), then these enzymes in our strain of E. coli B may be more sensitive to lower concentrations of ppGpp than those which affect RNA polymerase.

Lagosky and Chang (1980) have recently correlated the relationship between basal pool levels of ppGpp and RNA synthesis in E. coli (Hansen et al., 1975) using an analytical procedure by which the cellular levels of ppGpp is observed to be three to five times that shown by the acid extraction procedures. They discovered that RNA synthesis responded sensitively to changes in the ppGpp level in relA1 and spoT1 cultures during amino acid starvation and resupplementation. It is not clear how this relates to the apparent absence of such an effect on RNA synthesis in phase two cultures. The experimental conditions differed considerably between their study
and this one: the culture in the recycling fermentor is undergoing no abrupt, exogenously imposed transition in growth rate due to either amino acid or energy deficiency during phase two, and the observation interval, 22-24 h, is considerably longer than the 3 h maximum interval used by Lagosky and Chang (1980), or indeed of any other studies on cell responses to changing ppGpp levels to date.

These observations do not explain, however, why the phase two level of ppGpp is elevated above the basal level of the exponential growth of phase one. An important clue to understanding this observation can be found in the report of Braedt and Gallant (1976). They subjected the isogenic strains, NF161 and NF162 (relA1), to a glucose to succinate downshift and found that both ppGpp and cAMP accumulate, just the situation observed in E. coli B cells passing from the unrestricted carbon-energy growth of phase one to the glucose provision rate limited growth of phase two (Figures 8, 9). They found that the parent and the relA mutant accumulated maxima, respectively, of 1.63 and 0.35 nmoles ppGpp mg⁻¹ cell dry weight and 2.25 and 5.25 nmoles cAMP mg⁻¹ cell dry weight (calculated from their data) after the downshift. The maximum accumulations of ppGpp and cAMP that are observed in phase two E. coli B cells were 0.5 nmoles and 3.0 nmoles mg⁻¹ cell dry weight, respectively, similar to the levels found in NF162 (relA1), and corroborative of the RelA status of our strain.

In both a glucose or succinate downshift and in the transition from phase one to phase two, the glucose level in the milieu falls
below the transport level of the phosphotransferase (PTS) system, and since adenylate cyclase becomes active concurrent with the unsaturation of the PTS system (Botsford, 1981), the increased rate of production of cAMP is predictable for cells in either situation.

The interesting implication from both this work and Braedt and Gallant's results, however, is that some component of the ppGpp synthesizing system is also linked to the PTS system, becoming activated when the PTS system becomes glucose unsaturated. Since there is an increased ppGpp accumulation in NF162 (relA1) and in *E. coli* B (RelA) at the time of PTS unsaturation, it is likely that it is either the relX-relS, or spoT locus, or their products, which are affected.

The second peak in cAMP synthesis seen at the start of phase three (Figure 8) is not explicable in the same fashion since the PTS system is constantly in the same state of unsaturation that it was at the commencement of phase two. It may be that the depression of RNA synthesis at the start of phase three liberates ATP for use in cAMP synthesis.

An alternative set of implications arises when analyzing the effects of a nutrient upshift in *E. coli* on macromolecular synthetic rates, cell division rate, and ppGpp levels.

For example, immediately after a four-fold upshift in phase three, the growth rate becomes four times the rate of phase two rather than four times that of phase three (Figure 6 A, B), while the ppGpp level drops to about that of phase two. This indicates that the
rates of the major macromolecular syntheses are not correlated with the ppGpp level in phase 2 when it is below the critical level that invokes phase three, probably this occurs through restriction of rRNA synthesis. As suggested already, however, the morphological changes evident in phase two cells argue that the ppGpp levels in that phase are restricting cell elongation and septum formation, probably at the enzyme level. Electron micrographs (Figures 17, 18) of the many cells disrupted in the septal area after starvation, support the hypothesis that ppGpp is involved with inhibiting completion of septum formation in these cells, bringing about an indefinite extension of the D period of the cell cycle and postponement of cell division.

The increase in the rate of DNA synthesis, after an upshift of *E. coli* B, lagged the immediate increase in all other synthetic rates and the cell division rate by at least 10 h. This is the reverse of the situation usually observed in exponentially growing cells (Brunschede et al., 1977) when they are subjected to an energy or temperature upshift, where the change in cell division rate lags the change in DNA synthetic rate. This has been interpreted to mean that rounds of DNA synthesis underway when the shift is made must be completed before the rate of cell division can increase (Loeb et al., 1978) and that the rate of cell elongation is also fixed for the duration of any division cycle once started. The DNA content per cell in phase three (Figure 6 A, B, and Table 4) just prior to upshift was quite close to that reported by Tang and Helmhstetter (1980) in a
temperature-sensitive, DNA-initiation mutant of \textit{E. coli}, they accounted for the DNA enrichment in their culture as due to the presence of multiple chromosome forks. The DNA enrichment in \textit{E. coli} B may be due to multiple forks, or due to the presence of multiple copies of finished chromosomes, as either explanation fits the hypothesis that during phase three, the restriction of elongation and septum formation by ppGpp causes the accumulation of "primed sites" (Sloan and Urban, 1976) or a primed condition for cell elongation, septum formation, and cell division whose potential for completion is realized immediately after the upshift reduces the level of ppGpp below inhibitory concentrations and provides carbon-energy at a higher rate. Electron micrographs following an upshift (Figure 18), which revealed the presence of doubly dividing, or tripartite cells, argue strongly that envelope elongation and septum formation accelerate immediately, while DNA synthesis lags, probably completing the \(1 + C\) sequence of the cell cycle they were engaged in prior to the upshift. This phenomenon may be strain specific, since phase three cells of \textit{E. coli} H10407 become progressively smaller, and do not necessarily accumulate excessive quantities of DNA (Elfer, 1979) very similar to the situation in the \textit{E. coli} NF strains (Figure 15, Table 6).

Growth of \textit{B. polymyxa} before and after an upshift in GPR, is very much like that of \textit{E. coli} as the molar growth yield, \(Y\), of \textit{B. polymyxa} in the fermentor is independent of the specific growth rate, \(\mu\). Consequently, the relationship deduced by Pirt (1965) to exist between these quantities (and between \(Y\), ATP, and \(\mu\)) for chemostat
growth (equation 1), which is commonly used to evaluate maintenance energy demand again cannot be used for this purpose. Because μ decreases continuously in the recycling fermentor in phases two and three, while Y is constant within each phase, such a plot has a slope of zero, making the maintenance coefficient zero, and Y and Y_{max} equal. This is the same situation that exists in E. coli (Chesbro et al., 1979; Arbige and Chesbro, 1982), as the maintenance energy demand rate as defined in equation 1, is independent of cell mass within either phase two or three, rather than dependent on it as postulated in that formulation.

The molar growth yield in B. polymyxa changes abruptly at the transition point between phases two and three at the same time the effects of the stringent response appear, as it does in E. coli. The values of Y drop in phase two from a value near that of exponential growth to a lower value in phase three. Stringent regulation is, thus, a maintenance energy demand exactly as this concept is defined through equation 1, i.e. as a reduction in Y concurrent with the onset of the demand. More specifically, it is the consumption of ATP which does not lead to a net increase in biomass.

Two definitions of maintenance energy derived from different, basic considerations are in general use. By one definition, maintenance energy is the energy demand associated with maintaining the cell in a functional and viable condition (Dawes and Ribbon, 1964), and by a second definition is the energy utilized in the synthesis of new cell material. Neljssel and Tempest (1976) have
extended definition one to include energy spilling reactions. When cells are nutrient-limited by other than the energy source, i.e. are in energy excess, they spill energy in metabolite overflow, futile cycles, and other overload modulating reactions, rather than reduce their catabolic activity.

These authors pointed out that by leaving the capability for dissimilation unaltered in such conditions, the population maintained a potential for going immediately to a faster, or to a maximum growth rate if the nutrient limitation was removed. The energy thus spilled could be considered energy to maintain the cells growth potential.

Energy used both in sustaining cellular viability and population growth potential resolved the problem of classifying costs of such processes as chemotaxis and motility, costs proposed as maintenance energy costs but difficult to connect with maintenance of cell integrity (Dawes and Ribbons, 1964). These processes, however, clearly maintain growth potential by moving cells from less favorable environments.

Another basis for defining maintenance energy is through comparisons of growth yield (Y) when the growth rate is varied. The differences between the energy required to produce a unit of cell mass per unit time when Y has a maximum value and the energy similarly required when Y has some smaller value, i.e. when more energy is used in processes not yielding more biomass, has been inferred to represent the same energy of maintenance that is required to maintain cell viability and population growth potential.
Assuming then that all processes which use energy, without a net increase in biomass, are maintenance functions, in that they contribute to maintaining either cell integrity or growth potential, it is surprising to find little or no information concerning the cost of making regulatory nucleotides. Stouthamer (1979), however, did speculate that the energy demand of the stringent response was a maintenance cost, through a theoretical analysis of energy spilling mechanisms. This analysis would seem to include the cost of making all regulatory nucleotides, i.e. cAMP.

The results with *B. polymyxa*, using the convention of terming energy not producing new growth, maintenance energy, indicate that the cost of production and sustaining the level of regulatory nucleotides necessary to the stringent response in phase three cells is approximately 9% of the maintenance energy demand of these cells, while excretion and/or autolytic release of nucleic acid components and protein accounted for a further 8-11% of the demand. If *B. polymyxa* is similar to *E. coli* in that 51-58% of its maintenance energy demand is associated with membrane energization (Stouthamer and Bettenhaussen, 1977) than these three costs can account for 68 to 78% of the maintenance energy demand in phase three.

The cost of the stringent response, in addition to being an energy cost which does not produce growth, also can be defined as an energy cost that maintains the cell in a functional and viable condition. This is seen in the growth behavior of both *B. polymyxa* and *E. coli* following a four-fold upshift in phase three.
The overall growth rates immediately return to growth rates possessed 50-70 h earlier at the start of phase two, rather than a multiple of the growth rate it had immediately preceding the upshift. Thus, the cells anabolic potential was conserved during the period of extremely slow growth. O'Farrell (1978) has pointed out one of the most important consequences to the cell of the stringent response. The response is essential to preservation of the fidelity of translation during amino acid or energy deficiency conditions under which rel mutants lose viability more rapidly than do rel+ strains. Thus, the response conserves both cell viability, when involved, and growth potential. By analogy with the premise of Neljssel and Tempest (1976) the cost of the stringent response can then be categorized as energy expended to maintain anabolic potential.

The kinetics of starvation in E. coli B parallel responses of phase three cells of E. coli B to T4 virus infection (Evans, 1978), where a portion of the population (25-30%) immediately dies after each treatment, which is then followed by a 10 h stagnant period. After this period of constant viability, a second wave of death reduces the populations' viability by greater than 90%. The most obvious explanation for this phenomenon is seen in the electron micrographs of downshifted cells (Figures 17, 18). The first wave of death seems to be due to a loss of cell-hull integrity by cells in the septation phase of the cell cycle (D period). Since 30% of the population dies, it is likely that this portion of the population is in the D period, and must remain there for a sizeable fraction of the
better than 100 h division times. The remaining period of death appears to be due to the same cause, as the surviving cells can enter the septation phase of the cell cycle using any energy released from the first wave of bacterial lysis.

Growth characteristics of *Yersinia pestis* in the recycling fermentor, disregarding the apparent absence of an exponential growth period, resemble those of *E. coli* and *B. polymyxa* in that following a period of linear growth, a period of slower linear growth appears where RNA synthesis is depressed (Figure 23). This last period of linear growth resembles phase three in its entirety (Table 12). Charaetzky and Brubaker (1982) have recently reported that when *Y. pestis* strain EV76 is starved for calcium at 37°C, there is a reduction in the transcription of stable RNA synthesis which is not mediated by increased levels of guanosine tetra or pentaphosphate. In this report, they demonstrate that upon starving the cell for calcium, a small increase in guanosine tetraphosphate ensues, but that a much larger accumulation of guanosine tetraphosphate is evident when the cell is starved for phenylalanine. In the Ca²⁺-deprived cells, ppGpp levels were maintained for over 1 h before and 1 h after the alterations in RNA synthesis, but no attempt was made to analyze RNA synthesis in the amino acid starved culture. The authors felt that the delay in RNA restriction and the lower levels of ppGpp in the Ca²⁺-deprived cells, was evidence enough to conclude that the growth restriction was not arbitrated by (p)ppGpp. If a drop in RNA synthesis was delayed by 1 h in the amino acid starved
culture, though, the conclusions reached would seem to be invalid.

The onset of phase three, concurrent with the curbing of RNA accumulation is undoubtedly mediated by guanosine tetraphosphate (Arbige and Chesbro, 1982). It is apparent then that Y. pestis, at least in this energy limiting situation, does regulate the synthesis of RNA and biomass through the production of this special nucleotide.

The decision to use nalidixic acid as an "SOS" inducer in phase three cells of E. coli was both fortunate and unfortunate. Nalidixic acid is a specific inhibitor of DNA synthesis in proliferating cultures (Bauernfeind and Grummer, 1965), but neither inhibits DNA polymerase 1, endonuclease 1, or exonucleases 1, 11, and 111 from E. coli (Pedrini et al., 1972). In addition to inhibition of DNA synthesis, single-stranded breaks accumulate in the DNA (McDaniel et al., 1978). More specifically, nalidixate blocks the nicking and closing activity of the nalA gene, a component of DNA gyrase, which in turn leads to inhibition of DNA synthesis (Cozzarelli, 1980). Important features of the drug are that its effects are totally reversible, and it can be diluted out of the system (Guds and Pardee, 1976). Some other characteristics of this compound, which proved to be problematic to this study, included its ability to inhibit transcription (Gomez-Eichelmann, 1981), growth of bacteriophages (Baird et al., 1972), and colony forming ability (McDaniel et al., 1978). Nalidixic acid has also been used to define the link between inhibition of DNA synthesis and cell division (Miyakawa et al., 1980), as it blocks DNA synthesis immediately, but does not affect
the occurrence of cell division in D period cells (Donachie et al., 1971).

The drop in cellular enzyme levels after a nalidixic acid pulse in phase three (Figures 25, 28, 30), does not appear to be the result of an inhibition of transcription, since the enzymes were pulse-induced and the pulse has long since left the system as is evident by filtrate indole levels. Cell bound tryptophanase and β-galactosidase decay, following the nalidixate pulse, does not, however, prove that the recA protein, a protease, is either responsible for the decay or was undergoing similar degradation. Analogous experiments using a recC mutant of E. coli incapable of producing a functional exonuclease V, would disclose the link between recA protein and enzyme degradation, since nalidixic acid does not induce the production of recA protein in these mutants, although DNA synthesis is inhibited. Regardless of whether or not there is a direct link between the two, the phenomenon does exist and deserves further study.

The increase in colony forming units after nalidixate treatment (Figure 26) undoubtedly represent the number of cells in the process of septum formation. An increase in cell viability was noted by McDaniel et al. (1978) following the exposure of recA+ cells to nalidixic acid, but no such increase was obvious in recA mutants. The observation is further supported by the data in Table 11 which indicate that approximately 30% of phase three cells are in the D period. This is not to suggest that this method of residual division
is without error, since septum formation may not be completely blocked by chloramphenicol addition, due to the presence of pools of the required protein(s). In this situation some cells in the C period might escape the block and values of D would be overestimated. The decline in colony forming units after the rise is not explicable in the same way. It has been shown that colony forming ability decreases in the presence of nalidixate (McDaniel et al., 1978), and possibly the levels were not totally diluted out in the plating sequence of these cells. More likely, however, is that the newly divided daughter cells were susceptible to the stringent conditions of phase three, and rendered non-viable, possibly through a weakening of the cell wall components.

RecA protein is seemingly produced and active in phase three cells, as is evident through the increased synthesis of β-galactosidase following a nalidixate pulse in strain ATCC3311 which contains a lacZ gene fused to an operon under lambda repressor control (Figure 28). In this situation, however, the true enzyme levels are presumably much higher as nalidixic acid is probably both partially inhibiting transcription of the enzyme, and enhancing degradation simultaneously. This becomes a very important observation, as strains of this type are used for screening potential carcinogenic and carcinostatic agents (Elespuru and Yarmolinsky, 1979). If other agents that damage or distort DNA in higher organisms have the same degradative effects on the "screened enzyme", then many hazardous compounds would escape detection by this
method.

The increased rate of B-galactosidase synthesis seen after the nalidixate pulse has left the system, does indicate though that once the "SOS" system is turned on in these slow growing cells, it remains on in at least some of the cells for a very long time.

The burst of lambda phage following a nalidixate pulse (Figure 29) again provides evidence that an active RecA protein is produced in phase three. The inability to detect large numbers of virus was most likely due to a combination of adsorption and a reduced burst size as reported by Baird et al. (1972) when he treated cells with nalidixic acid. Through this experiment, however, it become apparent that a maximum of 38% of the cells in this phase three population produced an active recA protein, as all cells which release virus should be destroyed. A pulse of MMNG, though, affects the cells very differently, as colony forming units and biomass levels fell off sharply, reflecting substantial lysis of the population. These differing kinetics add proof that nalidixate affects only a certain portion of the phase three population (possibly C period cells), while the alkylating agent, N-methyl-N'-nitrosoguanidine has less cell cycle specificity and can induce RecA in the whole population.

The longevity of RecA, once induced, could not be exactly determined through these experiments, but the increased rate of B-galactosidase synthesis (Figure 28) seen hours after RecA had been induced argues that it is long-lived. Furthermore, if the protein behaves like B-galactosidase or tryptophanase, its rate of
degradation would be very slow indeed. Evans (1978) noted that B-galactosidase had a bimodal pattern of degradation, where the initial loss occurred at a much faster rate than the latter. This argued that B-galactosidase may be a protein whose degradation is energy dependent, or that two species of B-galactosidase exist, possibly periplasmic enzyme versus cytoplasmic enzyme. Tryptophanase on the other hand appeared to be an extremely stable enzyme, whose level once stabilized remained relatively unaltered for up to 40 h (Figure 28), arguing again that breakdown of this protein might be energy dependent.

If either B-galactosidase or tryptophanase, whose patterns of degradation are somewhat different, are a reliable index of protein loss in these energy limited cells, then RecA would be very stable in this system.


