BIOSYNTHESIS AND REGULATION IN ESCHERICHIA COLI B GROWN IN THE MAINTENANCE ENERGY FERMENTER

THOMAS MORGAN EVANS

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UNIVERSITY OF NEW HAMPSHIRE, PH.D., 1978

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BIOSYNTHESIS AND REGULATION IN *ESCHERICHIA COLI* B GROWN IN THE MAINTENANCE ENERGY FERMENTER

BY

THOMAS MORGAN EVANS

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Doctor of Philosophy

in

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ABSTRACT

BIOSYNTHESIS AND REGULATION IN ESCHERICHIA COLI B
GROWN IN THE MAINTENANCE ENERGY FERMENTER

by

THOMAS MORGAN EVANS

An anaerobic continuous culture device with biomass feedback has been developed to investigate the limit conditions in a culture of Escherichia coli B when the energy source is the limiting nutrient. Growth in this system consisted of three sequential phases: exponential growth (phase 1), substrate dependent growth (phase 2) and the maintenance energy phase (phase 3). Theoretical analysis of mass transfer in this system predicted the occurrence of three phases. Phase 1 growth and the limit of this growth were in agreement with the predicted pattern of growth in this phase. The growth in phases 2 and 3 deviated from that which was predicted. Phase 2 growth was linear in nature whereas the predicted growth was hyperbolic with the growth rate decreasing as the maintenance energy requirements of the population increase. The observed growth in phase 3 was in direct contrast to the predicted steady state where the ultimate constant mass in the culture system was postulated to be determined by the populations' maintenance energy requirements.
The observed constant rate of growth in phases 2 and 3 was postulated to be the result of a continuously decreasing maintenance coefficient. A cellular process which would allow the maintenance coefficient to continuously decrease is one means by which a culture can regulate the amount of energy used for growth and that used for maintenance. This has important consequences when considering the observation that a culture is unable to use all of the available energy for maintenance purposes and thereby reduce the growth rate in phase 3 to zero.

Phases 2 and 3 were found to be periods of unbalanced growth where phase 2 cells became reduced in percent composition of RNA and enriched in protein and DNA and phase 3 cells became reduced in percent composition of DNA and RNA and enriched in protein. The unbalanced growth was considered to be a result of stringent control of RNA, phospholipid and lipopolysaccharide synthesis as well as the blockage of the incorporation of DNA precursors into DNA. The process of protein enrichment in phases 2 and 3 may be the result of an accumulation of a type of protein whose loss is energy dependent and would accumulate under conditions of energy restriction.

The inducibility of the lactose operon in phases 2 and 3 was an indication that the traditional operon controls were functional in the two phases. The capacity for induction of the lactose operon when the energy supply was glucose was thought to be due to the low intracellular concentrations of glucose during phases 2 and 3. The concentration of glucose
at very low levels would be insufficient to mediate catabolite repression.

Reduced rates of B-galactosidase synthesis during a second induction, in a culture which was induced, allowed to become repressed and then re-induced was taken for evidence of "aging" in the protein synthesizing capability in phase 3. The differences between the rate of loss of protein and B-galactosidase during the different phases indicated that B-galactosidase was not a good indication of the overall rate of protein loss.

The pattern of a phase 3 culture infection with bacteriophage T4 indicated that a maintenance energy phase culture very tightly controls the use and allocation of intracellular energy supplies.
ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. William Chesbro for his guidance and many helpful suggestions during the course of this investigation. I am also grateful for the many helpful discussions with Dr. Robert Zsigay.

I would also like to sincerely thank Ms. Robin Eifert for her help in operating the maintenance energy fermenter and for conducting many of the cellular assays and Dr. David Balkwill for the electron micrographs.

But most of all I wish to thank my wife, Rosemary, for her encouragement and support.
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CHAPTER I
INTRODUCTION

A. The Concept of Energy of Maintenance

The concept of maintenance energy has been a nebulous one since its inception. Intuitively, the idea that microorganisms require energy to maintain the status quo, i.e., require energy for functions other than those associated with growth, is a significant one. Early investigations into microbial growth indicated that energy may be required for overcoming the effects of chemical wear and tear, in ion transport and in lending stability to macromolecules, and may be wasted in energy uncoupling (Mallette, 1963). In later investigations the energy expended in these processes would be recognized as energy of maintenance. Duclaux in 1898 was probably the first microbiologist to distinguish between energy for growth and energy of maintenance (Pirt, 1965). He subdivided the substrate metabolized by a culture into two categories, the energy source consumed per amount of organism formed and the amount of energy source consumed per amount of organism per time to maintain the organism in a healthy state.

Since Duclaux's work most investigations concerning maintenance energy searched for direct experimental evidence for this concept. The investigations of Monod (1942) indicated that the energy of maintenance in growing cultures was
essentially zero. The rationale used as a basis of Monod's experiments was that if exogenous energy substrates could be utilized as a source of maintenance energy, then there should be a concentration of energy source at which growth would not occur and the energy substrate would be used only for the maintenance of the cell. Therefore, experimental evidence for maintenance energy could be deduced from an extrapolation of a plot of turbidity versus exogenous energy source concentration to zero turbidity. An intercept at a finite concentration of the energy source would validate the concept of maintenance energy. In experiments with both Escherichia coli and Bacillus subtilis, Monod (1942) found that a plot of turbidity versus substrate concentration, extrapolated through the origin and concluded that maintenance energy did not exist. Similar experiments were conducted by other investigators using Aerobacter (Enterobacter) aerogenes and Saccharomyces cerevisiae and their conclusions about maintenance energy were identical to Monod's - maintenance energy did not exist (Dagley et al., 1951; Bauchop and Elsdon, 1960).

A reconsideration of the question concerning the existence of maintenance energy was a consequence of the development of continuous culture. Herbert (1958) in his discussion of the theoretical principles of continuous culture concluded that the experimentally observed curve relating steady-state bacterial concentration to specific growth did not coincide with the predicted curve. He found at low specific growth rates (equation 5) the cell yield (amount of biomass formed per unit amount of substrate consumed) was less than expected
from a theoretical consideration of specific growth rate and cell yield. This difference he explained by hypothesizing that a certain amount of biomass was consumed endogenously to meet the maintenance energy demands of the population. This pattern of reduced cell yield at low specific growth rates was later confirmed by Schulze and Lipe (1964). They also hypothesized that processes contributing to energy of maintenance were responsible for the reduced cell yield.

Further evidence for energy of maintenance came from experiments which re-examined the hypothesis that Monod developed concerning the extrapolation of plots of growth vs substrate concentration to zero growth. The experiments of McGrew and Mallette (1962) were designed with increased sensitivity to permit comparison of growth with exogenous energy source by lowering the level of energy source relative to cell concentration. They were able to find a substrate concentration such that when the substrate was added to the culture at regular intervals, the turbidity of the culture remained constant. The lack of change indicated that exogenous energy was metabolized without growth. A decrease in culture viability, as measured by plate counts during the period of glucose addition, indicated that glucose feeding was not as effective in maintaining viability as it was in maintain turbidity. The authors hypothesized that cell clumping may have contributed to the decrease in cell numbers. Also, periodic addition of the energy source as opposed to constant addition may have contributed to loss of cell numbers. They concluded, on the basis of the above evidence, that energy
of maintenance did exist. Possibly the reason that Monod (1942) and others (Dagley et al., 1951; Bauhop and Elsden, 1960) did not find evidence for the existence of energy of maintenance is that the growth yield in unrestricted culture (batch culture) is considerably less than in restricted growth (fed-batch or continuous culture) (Marr et al., 1963). The reduced growth yields in the batch cultures may have masked any maintenance requirements (Marr et al., 1963).

The approach used by McGrew and Mallette (1962) to investigate maintenance energy in which cultures were fed energy substrate at regular intervals was continued in the experiments of Marr, Nilson and Clark (1963). These authors considered maintenance energy as a process independent of growth rate. They derived an equation relating growth rate and maintenance energy to the change in substrate concentration. The relationship was based on the assumption that bacterial growth occurs at a linear rate proportional to the rate of substrate utilization. The equation relating bacterial growth to substrate utilization is:

\[ \frac{dx}{dt} = Y ds \]

where \( x \) is the concentration of cells, \( Y \) is the yield coefficient or the weight of cells produced per unit weight of limiting nutrient, \( S \) is the concentration of limiting nutrient and \( t \) is time. Equation 1 was modified to include maintenance energy demands and was restated as:

\[ \frac{dx}{dt} + aX = \frac{Y ds}{dt} \]
where the term $aX$ represents, in the authors' words, the amount of substrate diverted from growth and $a$ is the specific maintenance rate expressed in $(h)^{-1}$. The relationship stated in equation 2 was tested by feeding four identical exponential (batch) cultures of *E. coli* the same amount of energy substrate at four different rates. One culture (control) was fed the ration of glucose all at one time. The three remaining cultures were fed the energy substrate at three rates. A comparison of the culture which was continuously fed substrate with the culture which was fed the substrate ration instantaneously indicated that growth was nearly linear as predicted by equation 1. Similar comparisons of the cultures fed glucose slowly indicated growth was curvilinear as predicted by equation 2. On the basis of these experiments, Marr, Nilson and Clark concluded that maintenance energy was a valid concept and were able to obtain a value of 0.028 $(h)^{-1}$ for the specific maintenance rate. Since one value of the maintenance rate used in equation 2 could describe the curvilinear growth, the authors concluded that the specific maintenance rate was independent of growth rate.

The concept of maintenance energy was further developed to explain observations of lowered cell yields resulting from continuous culture growth at low specific growth rates. Herbert (1958) postulated that endogenous metabolism, defined as the total metabolic reactions that occur within the cell in the absence of a usable exogenous energy substrate (Dawes and Ribbons, 1964), accounted for the reduced cell yields. The endogenous metabolism that Herbert referred to is identical
to maintenance energy and was incorporated into the growth rate law as:

$$\frac{dx}{dt} = (b-a)X$$  \hspace{1cm} (3)

where $X$ is the cell concentration, $t$ is time, $b$ is the specific growth rate and $-ax$ represents the loss of cell biomass due to maintenance. This relationship was also adopted by Marr et al. (1963) and they termed the rate constant, $a$, the specific maintenance rate. Marr et al., modified equation 3 to describe energy limited growth in continuous culture and obtained:

$$\frac{1}{X} = \frac{a}{X_{\text{max}}} + \frac{1}{D} + \frac{1}{X_{\text{max}}}$$ \hspace{1cm} (4)

Where $x$ is cell concentration, $X_{\text{max}}$ is the maximum organism concentration and $D$ is the dilution rate. The dilution rate is defined as the flow rate (volume)$^{-1}$ and determines the specific growth rate of cells growing in continuous culture. At a steady state mass concentration in continuous culture, the specific growth rate will be equal to the dilution rate (Herbert et al., 1956). Specific growth rate is the rate of increase per unit of organism concentration and is expressed as:

$$\mu = \frac{1}{X} \left( \frac{dx}{dt} \right)$$ \hspace{1cm} (5)

where $\mu$ is the specific growth rate and has the dimension of (h)$^{-1}$. The relationship expressed in equation 4 was tested by growing bacteria at different dilution rates in continuous culture and plotting $1/X$ vs $1/D$. This double reciprocal plot was found to be linear which indicated the assumption made in equation 4 was a valid one.
The concept of maintenance energy as applied to continuous culture by Herbert (1958) was also verified by Schultze and Lipe (1964) and Pirt (1965). Schultze and Lipe, as well as Pirt, viewed the energy necessary for maintenance of cells growing in continuous culture as a diversion of a part of the energy derived from the metabolism of an exogenous energy source. Up to this time, the source of energy for maintenance was hypothesized to be supplied from the consumption of endogenous biomass (Herbert, 1958; Marr et al., 1963; Dawes and Ribbons, 1964). Schultze and Lipe approached the mathematics of maintenance energy as Herbert did, except they represented maintenance energy as grams of substrate consumed per gram cell biomass per hour. They also recognized that maintenance energy demands would influence the value of the yield coefficient (cell yield). They obtained a yield coefficient corrected for maintenance energy from a plot of the reciprocal of the yield coefficient versus dilution rate. The intercept of the curve at the ordinate represented the value of the yield coefficient corrected for maintenance energy consumption of substrate. Schultze and Lipe related maintenance energy defined in terms of substrate consumed to Herbert's concept of maintenance defined as a consumption of endogenous materials by the relationship:

\[ m = \frac{a}{Y_G} \]

where \( m \) is energy consumed for maintenance purposes expressed in energy substrate consumed (unit mass of culture)\(^{-1}\) (unit time)\(^{-1}\) and \( Y_G \) is the yield coefficient corrected for maintenance energy.
Pirt (1965) formalized the theories of maintenance energy into the substrate balance where:

overall rate of substrate utilization = rate of substrate utilization for growth + rate of substrate utilization for maintenance

Expressed mathematically, the above expression becomes:

\[
\frac{ds}{dt} = (\frac{ds}{dt})_M + (\frac{ds}{dt})_G
\]

Using the following substitutions:

\[
ds/dt = -\mu x/Y; (ds/dt)_M = -mx; (ds/dt)_G = -\mu x/Y_G
\]

Pirt derived the formula:

\[
\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_{\text{max}}}
\]

where \(Y_{\text{max}}\) is equivalent to \(Y_G\) and is the maximum theoretical growth yield when maintenance energy is zero. Pirt defined \(m\) as the "maintenance coefficient" expressed in energy substrate consumed (unit mass of culture\(^{-1}\) unit time\(^{-1}\). The maintenance coefficient is identical to the term, energy consumed for maintenance, used by Schulze and Lipe.

From the above discussion, it is apparent that the energy supply for maintenance may be derived by either of two hypothetical mechanisms, that of Pirt (1965) where maintenance energy requirements are met by catabolizing absorbed substrate and that of Herbert (1958) where maintenance requirements are met by catabolizing endogenous biomass. Sykes (1976) considered the merits of these two hypotheses in some detail.
Sykes hypothesized that since cellular biomass captures only 55% of the exogenous substrates' energy and carbon, a cell meeting its maintenance energy demands by catabolizing endogenous biomass would ultimately use about twice as much substrate as would a cell meeting its maintenance energy demands by the direct metabolism of exogenous energy substrate. Therefore, Sykes concluded that the model of Pirt was more sound from the biochemical viewpoint. Sykes further tested the models of Pirt and Herbert by developing substrate balances based on either Pirt's or Herbert's analysis of growth yield and applying these balances to observed growth in continuous culture. The substrate balance included terms for substrate input and washout, substrate used in synthesis, substrate converted to excreted metabolite and in the balance which tested Pirt's theory, substrate expended for maintenance. Sykes found that only the balance that included consumption of exogenous substrate for maintenance adequately described growth when all terms of the substrate balance were considered. Therefore, Sykes concluded that the model of Pirt was the correct one.

B. Magnitude and Distribution of Maintenance Energy in Microorganisms.

Energy of maintenance is defined as the energy expended by cellular processes that are not directly associated with growth (Mallette, 1963). The amount of energy used for growth will depend on the nature of the organism's metabolism and on the cultural conditions under which it is grown. The variation in the amount of energy used for maintenance purposes
in various microorganisms is shown in Table 1. The values of the maintenance coefficient ($m_{ATP}$) ranged from 0.21 and 0.25 (mmole ATP) (g dry biomass)$^{-1}$ (h)$^{-1}$ for the yeasts, *Candida parapsilosis* and *Saccharomyces cerevisiae*, respectively, to 220 (mmole ATP)(G dry biomass)$^{-1}$ (h)$^{-1}$ for *Azotobacter vinelandii* when fixing nitrogen at a high dissolved oxygen tension. Stouthamer and Bettenhausen (1973) calculated for *E. aerogenes* growing anaerobically, with glucose in excess, at a specific growth rate of 0.1 (h)$^{-1}$ that approximately 90% of the total ATP produced was used for maintenance. This calculation indicates that under certain growth conditions the energy expended for maintenance can be a major component of the cell's total energy budget.

Culture conditions dramatically influence the magnitude of the maintenance coefficient (Table 1).

C. **Cellular Processes Which Contribute to Energy for Maintenance.**

Various authors have assessed the effects of culture conditions on maintenance energy and which cellular processes contributed to maintenance energy functions.

Marr, Nilson and Clark (1963) investigated the effect of galactoside transport on energy of maintenance. The experimental rationale used in their experiments was that the energy required for the accumulation of the galactoside against a concentration gradient should divert metabolic energy from growth processes and increase the maintenance energy requirements. A comparison of the maintenance rates in *E. coli* growing in fed-batch cultures either in the presence or absence of the
Table 1. Distribution of Maintenance Energy Requirements in Microorganisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth Condition</th>
<th>Maintenance Energy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobacter (Enterobacter) cloacae</td>
<td>aerobic, glucose-limited</td>
<td>0.094</td>
<td>Stouthamer &amp; Bettenhaussen 1973 &amp; 1975</td>
</tr>
<tr>
<td>Aerobacter (Enterobacter) aerogenes</td>
<td>anaerobic, glucose-limited</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>anaerobic, complex medium</td>
<td>-</td>
<td>Stouthamer, 1977</td>
</tr>
<tr>
<td></td>
<td>anaerobic, tryptophan-limited</td>
<td>2.88</td>
<td>Stouthamer, 1977</td>
</tr>
<tr>
<td></td>
<td>anaerobic, citrate-limited</td>
<td>-</td>
<td>Stouthamer, 1977</td>
</tr>
<tr>
<td></td>
<td>aerobic; glucose-limited</td>
<td>0.3</td>
<td>Stouthamer, 1977</td>
</tr>
<tr>
<td>Azotobacter vinelandii</td>
<td>fixing nitrogen, dissolved oxygen</td>
<td>1.5</td>
<td>Nagai &amp; Aiba, 1972</td>
</tr>
<tr>
<td></td>
<td>tension 0.2 atm</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td></td>
<td>fixing nitrogen, dissolved oxygen</td>
<td>0.15</td>
<td>Nagai &amp; Aiba, 1972</td>
</tr>
<tr>
<td></td>
<td>tension 0.02 atm</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>anaerobic, glucose-limited</td>
<td>-</td>
<td>Rogers &amp; Stewart, 1974</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>anaerobic, glucose-limited</td>
<td>-</td>
<td>Hempfling &amp; Mainzer, 1975</td>
</tr>
<tr>
<td></td>
<td>aerobic, glucose-limited</td>
<td>18.9</td>
<td>Schulze &amp; Lipe, 1964</td>
</tr>
<tr>
<td></td>
<td>aerobic, glucose-limited</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>aerobic, glucose-limited</td>
<td>0.4</td>
<td>Hempfling &amp; Mainzer, 1975</td>
</tr>
</tbody>
</table>
Table 1. continued

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth Condition</th>
<th>Maintenance Energy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactobacillus casei</strong></td>
<td>anaerobic, complex medium, glucose-limited</td>
<td>0.135 1.5</td>
<td>deVries et al., 1970</td>
</tr>
<tr>
<td><strong>Micrococcus denitrificans</strong></td>
<td>aerobic, gluconate-limited</td>
<td>- 0.53</td>
<td>VanVerseveld &amp; Stouthamer, 1976</td>
</tr>
<tr>
<td><strong>Penicillium chrysogenum</strong></td>
<td>aerobic, glucose-limited</td>
<td>0.22 3.2</td>
<td>Righelato et al., 1968</td>
</tr>
<tr>
<td><strong>Pseudomonas sp</strong></td>
<td>aerobic, methane-limited</td>
<td>1.9 -</td>
<td>Nagai, Mori &amp; Aiba, 1973</td>
</tr>
<tr>
<td><strong>Saccharomyces cerevisiae</strong></td>
<td>anaerobic, glucose-limited</td>
<td>0.036 0.52</td>
<td>Watson, 1970</td>
</tr>
<tr>
<td>****</td>
<td>anaerobic, glucose-limited</td>
<td>- 0.25</td>
<td>Rogers &amp; Stewart, 1970</td>
</tr>
<tr>
<td>****</td>
<td>anaerobic, glucose-limited, NaCl (1.0M)</td>
<td>0.360 2.2</td>
<td>Watson, 1970</td>
</tr>
<tr>
<td><strong>Thiobacillus neapolitanus</strong></td>
<td>aerobic, thiosulfate-limited</td>
<td>3.44 -</td>
<td>Hempfling &amp; Vishniac, 1967</td>
</tr>
</tbody>
</table>

\[ \text{Mc}, \text{ Maintenance coefficient expressed as g energy source (g dry biomass)}^{-1}(\text{h})^{-1}. \]
\[ \text{M}_{\text{ATP}}, \text{ Maintenance coefficient expressed as mmole ATP (g dry biomass)}^{-1}(\text{h})^{-1}. \]
galactoside, methyl-β-D-thiogalacto-pyranoside, indicated that active transport of this solute did not contribute or increase the maintenance energy requirements. Marr et al. also determined the effect of temperature on the value of the maintenance rate. Using fed-batch cultures, they found that the maintenance rate of E. coli grown at 30°C decreased from 0.028 (h)^{-1} to 0.005 (h)^{-1} when grown at 15°C.

The effect of temperature on maintenance energy has also been examined during continuous culture growth of E. coli B (Mainzer and Hempfling, 1976). As did Marr et al., Mainzer and Hempfling observed a relationship between temperature and maintenance energy. These authors found that during anaerobic continuous culture of E. coli B the value of the maintenance coefficient decreased by 25% when the growth temperature was decreased from 30°C to 25°C. The maintenance coefficient referred to by the authors was m_{ATP} which is defined as the maintenance coefficient expressed in mmol ATP(g dry wt)^{-1}(h)^{-1} instead of mmol or g energy substrate (gram dry wt)^{-1}(h)^{-1}.

The value of m_{ATP} is calculated by the same means as is m_c except the yield coefficient (Y_A) is expressed as Y_{ATP} where Y_{ATP} is the yield coefficient expressed in terms of ATP(unit biomass)^{-1} instead of energy substrate(unit biomass)^{-1} (Stouthamer, 1969). The value of Y_{ATP}^{max} is obtained from the ordinate intercept of a double reciprocal plot of Y_{ATP} vs D. The authors also found that Y_{ATP}^{max} increased from 10.3 g bacterial dry weight(mmol ATP)^{-1} to 12.7 g bacterial dry weight(mmol ATP)^{-1} when the growth temperature decreased from 37°C to 25°C. A similar relationship between both the maintenance respiration
rate and growth yield and temperature was found in aerobic chemostat cultures of \textit{E. coli} B. The maintenance respiration rate from 37° to 32°C increased from 0.9 to 4.4 and then decreased to 1.5 as the temperature was lowered to 17.5°C. In aerobic culture, the maintenance respiration rate \( m_0 \) is defined as mg-atom of O(g bacterial dry wt)\(^{-1}\)(h)\(^{-1}\) and the growth yield is defined as g bacterial dry weight(g-atom O)\(^{-1}\) (Stouthamer, 1969). The authors were unable to explain the observed phenomenon where both \( Y_Q \) and \( Y_{ATP} \) increased and both \( m_0 \) and \( m_{ATP} \) decreased when growth temperature was decreased. They hypothesized that the efficiency of biomass synthesis may increase at lower growth temperatures.

The effects of culture pH on \( m_c \) have not been systematically examined. Harrison and Loveless (1971) did observe a decrease in growth yield with a decrease in culture pH. Pirt (1975) calculated from the data of Harrison and Loveless that the observed decrease in growth yield, when the culture pH decreased from 6.6 to 5.4, would result in an increase in the value of the maintenance coefficient.

The relationship between the nature of the limiting energy substrate and maintenance energy in aerobic continuous culture of \textit{E. coli} B was examined by Hempfling and Mainzer (1975). The authors found that the magnitudes of both \( Y_0^{\text{max}} \) and \( m_0 \) were a function of the identity of the substrate which limited growth. The authors hypothesized that the dependence of \( m_0 \) and \( Y_0^{\text{max}} \) on the identity of the growth limiting substrate may be a result of changes in efficiency of oxidative phosphorylation with the identity of the substrate. The dependence
of the magnitude of the maintenance coefficient on the identity of the substrate was also verified in chemostat cultures of Micrococcus denitrificans (Van Verseveld and Stouthamer, 1976) and of E. aerogenes (Stouthamer and Bettenhausen, 1975).

The concentration of dissolved oxygen in aerobic chemostats has been shown to influence the rate at which a substrate is used for maintenance (Rogers and Stewart, 1974; Nagai and Aiba, 1972).

The nature of growth limitation in chemostat cultures has a marked influence on the magnitude of the maintenance coefficient. In chemostat culture where the limiting nutrient (tryptophan, nitrogen, phosphate or sulfate) was not the energy source, the maintenance coefficient was greater than when the identical organism was grown under energy limitation (Neijssel and Tempest, 1976a, 1976b, and Stouthamer and Bettenhausen, 1975).

The cellular processes which comprise the largest fraction of the maintenance energy requirement were thought to be protein turnover (Hempfling and Mainzer, 1975; Marr et al., 1963) and preservation of the proper ionic composition of the cell (Stouthamer and Bettenhausen, 1973). Marr, Nilson and Clark (1963) in their studies of maintenance energy in the fed-batch culture system calculated what fraction of the maintenance rate was due to protein turnover. They found in an aerobic culture of E. coli B, where growth was restricted by feeding glucose, that protein was degraded in the culture at a rate of 0.032 (h)^{-1}. Based on the amount of substrate necessary to replenish protein loss, Marr et al. calculated a
maintenance rate of 0.016 (h)^{-1} or about 60% of the measured maintenance rate of 0.028 (h)^{-1} was due to protein turnover. Similar calculations were made by Hempfling and Mainzer (1975). They assumed a protein turnover rate of 0.025 (h)^{-1} for an exponential culture of *E. coli* B and calculated that approximately 50% of the maintenance energy requirements of cells growing aerobically on glucose was due to protein turnover. The idea that protein turnover comprises 50% of the maintenance energy requirements is not universally accepted. Pirt (1975) concluded that protein turnover comprises only 6% of the maintenance requirements in exponentially growing *E. coli*. The value \( \sqrt{0.006}(h)^{1/2} \) of protein turnover that Pirt used to calculate the fraction of maintenance energy due to protein turnover was substantially lower than the value used by Marr et al., \( \sqrt{0.032}(h)^{1/2} \) and Hempfling and Mainzer \( \sqrt{0.025}(h)^{1/2} \). The lack of agreement between values or protein turnover used in the calculation of the protein fraction of maintenance energy would account for the different conclusions reached by the various authors as to the fraction that protein comprises of the maintenance energy requirements.

Another cellular process which is thought to contribute in a large way to maintenance energy requirements is the maintenance of the proper ionic balance in the cell. Watson (1970) determined the value of \( m_c \) in aerobic glucose-limited continuous cultures of *S. cerevisiae* in the presence or absence of 1.0 M NaCl. The value of \( m_c \) increased from 0.2 \( \mu \text{mol glucose (mg dry weight)}^{-1}(h)^{-1} \) in the absence of NaCl to 2 \( \mu \text{mol glucose (mg dry weight)}^{-1}(h)^{-1} \) in the presence of NaCl. Stouthamer
and Bettenhausen (1973) found similar effects of increased extracellular salt concentration on the maintenance coefficient. They report an increase of $m_{\text{ATP}}$ from $0.0387 \text{ g mole ATP(g dry weight)}^{-1}(\text{h})^{-1}$ to $0.050 \text{ g mole ATP(g dry weight)}^{-1}(\text{h})^{-1}$ when E. aerogenes was grown in continuous culture in the presence of a high NH$_4$Cl concentration. Both Watson and Stouthamer and Bettenhausen concluded that a large fraction of the energy for maintenance was used for the preservation of the proper ionic composition of the cell.

In several species of Bacillus, such as B. subtilis and B. licheniformis, autolysis has been observed in the absence of continued cell wall synthesis for which expenditure of ATP is necessary (Shockman et al., 1961). For this reason, Stouthamer and Bettenhausen (1973) postulated that in Bacillus species cell wall turnover may comprise a large fraction of the maintenance energy budget.

Energy expended for maintenance may also be a result of energy-uncoupled growth (Stouthamer and Bettenhausen, 1975). Energy uncoupling was defined by Senez (1962) as a lack of regulating the energy-yielding metabolism to the energy-consuming reactions of cell biosynthesis. Energy uncoupling may occur in cultures growing under unfavorable conditions. Growth at a suboptimal temperature, in a medium in which one or more components are present at suboptimal amounts, under nitrogen or phosphate limitation or in the presence of inhibitors may lead to energy uncoupling (Stouthamer and Bettenhausen, 1975; Senez, 1962). Energy uncoupling has been postulated to explain the large maintenance coefficient found in A. vinelandii when
fixing molecular nitrogen at high oxygen tensions (Nagai and Aiba, 1972). This high maintenance coefficient is thought to be a result of the energy expended in the protection of the nitrogenase system from oxygen (Pirt, 1975).

The production of secondary metabolites, such as penicillin by P. chrysogenum, has been considered as a maintenance energy process (Righelata, Trinci and Pirt, 1968).

Since energy required for the development of an energized membrane may require as much as 60% of the energy produced by respiring anaerobic E. coli, this process may contribute in a large way to the energy used for maintenance (Stouthamer and Bettenhausen, 1977).

D. The Relationship Between Growth Rate and Energy of Maintenance.

One of the consequences of formulae developed by Pirt (1965), equation 7, and Marr et al. (1963), equation 4, is that the energy required for maintenance (m_c) be independent of growth rate. Just as these authors found that the relationship held true over a range of growth rates, several authors have found that these relationships are not valid for certain organisms and for certain conditions of growth (deVries et al., 1970; Hobson and Summers, 1967; Carter et al., 1971; Stouthamer and Bettenhausen, 1975). The lack of linearity of the double reciprocal plot of Y_a versus \( \mu \) was shown to be due to the influence of \( \mu \) on the energy yielding metabolism and on the ATP yield of the organism.
Stouthamer and Bettenhaussen (1975) found a much larger $M_{\text{ATP}} \frac{0.0387}{(\text{mol ATP (g dry weight)}^{-1}(\text{h})^{-1}}$ in tryptophan-limited anaerobic chemostat cultures of $E. \text{aerogenes}$ than in glucose-limited cultures $\frac{0.0068}{(\text{mol ATP (g dry weight)}^{-1}(\text{h})^{-1}}$. They concluded that part of the increased maintenance energy requirements in the tryptophan-limited culture was due to growth-dependent processes which were not associated with the formation of new cell material. They postulated that these growth-dependent processes were not true maintenance functions.

Investigations concerning carbon-limited and carbon-sufficient chemostat cultures of $K. \text{aerogenes}$ has indicated that magnitude of the maintenance coefficient was not independent of growth rate (Neijssel and Tempest, 1976a). Results of these experiments indicated that the maintenance rate increased 2.5-fold over the range of growth rates ($u$) from zero to 0.5 (h)$^{-1}$. Neijssel and Tempest concluded that the maintenance rate could conceivably increase with growth rate since fast growing organisms contain a higher intracellular concentration of osmotically active molecules ($K^+$, $Mg^{2+}$, and glutamate ion) and a greater content of labile macromolecules, such as RNA, than do slower growing organisms.

As a result of the evidence that indicated the maintenance coefficient was not independent of growth rate, the original equation of Pirt (1965) was modified to correct for growth-dependent maintenance processes. Stouthamer and Bettenhaussen (1973) derived the equation:
where $q_{\text{ATP}}$ is the specific rate of ATP production expressed as mol ATP(g dry weight)$^{-1}$(h)$^{-1}$, $Y_{\text{ATP}}^{\text{max}}$ is the growth yield per mol ATP corrected for energy of maintenance and $m_e$ is the maintenance coefficient expressed as mol ATP(g dry weight)$^{-1}$(h)$^{-1}$. Equation 9 indicates that $q_{\text{ATP}}$ is a linear function of $\mu$ and that $Y_{\text{ATP}}$ is dependent on the growth rate. This relationship has been observed for L. casei, S. cerevisiae, E. aerogenes and E. coli (Stouthamer, 1977).

The relationship between growth and oxygen consumption in aerobic chemostats was modified by Neijssel and Tempest (1976a) to allow the maintenance rate of oxygen consumption to vary with growth rate. The authors assumed that if the maintenance rate varied with growth rate it did so in a linear fashion, and derived the equation:

$$\frac{1}{Y_{O_2}} = \frac{1}{Y_{O_2}^{\text{max}}} + cq_{O_2}^m + q_{O_2}^m$$

where $Y_0$ is growth yield per amount of oxygen consumed (g dry weight per mol O$_2$), $Y_{O_2}^{\text{max}}$ is the growth yield per mol O$_2$ corrected for maintenance, $c$ is the constant that defines the variation in maintenance rate with growth rate and $q_{O_2}^m$ is maintenance coefficient expressed in mol O$_2$ (g dry weight)$^{-1}$(h)$^{-1}$. 
E. Theoretical Basis of the Maintenance Energy Fermenter.

Schultz and Gerhardt (1969) in their review of dialysis culture considered four modes of operation in which nutrients were supplied to the culture through a dialyzing membrane. The characteristics of one of these configurations (Figure 1a), with modification, can be used to describe the operating properties of the maintenance energy fermenters. The authors were able to show that growth in dialysis system could be described by the relationship:

\[
\frac{dx}{dt} = \frac{Y_x P_M A_M (S_R - S_F)}{V_F} - Y_E X X
\]

where \(X\) is the cell concentration in g/ml\(^{-1}\), \(Y_x\) is the yield coefficient for conversion of substrate to cells in g of cells (g of substrate)\(^{-1}\), \(P_M\) is the membrane permeability coefficient for substrate in cm(h)\(^{-1}\), \(A_M\) is the area of the membrane in cm\(^2\), \(S_R\) is the glucose concentration in the reservoir chamber in g/ml\(^{-1}\), \(S_F\) is the glucose concentration in the fermentation chamber in g/ml\(^{-1}\), \(V_F\) is the fermentation chamber volume in ml and \(Y_E\) is the specific maintenance rate per h. The second term in the equation represents the amount of cell biomass consumed endogenously to supply the maintenance demands of the growing population. Since a dimensional analysis of the term \(Y_E X X\) indicated that the term represents glucose consumed (ml)\(^{-1}\)(h)\(^{-1}\), the authors have misstated the equation. If the maintenance coefficient \(M_c\) is substituted for \(Y_E\), the term would then represent consumption of biomass to fulfill the maintenance demands. Substitution of \(M_c\) for \(Y_E\) in equation 10
Fig. 1a. Diagrammatic representation of the dialysis culture apparatus of Schultz and Gerhardt (1969) showing the defining characteristics; fermenter volume ($V_p$) in ml, fermenter substrate concentration ($S_p$) in g(ml)$^{-1}$, cell concentration ($X$) in g(ml)$^{-1}$, reservoir volume ($V_R$) in ml, flow rate ($F_p$) in ml(h)$^{-1}$, substrate concentration in fed stream ($S_p^0$) in g(ml)$^{-1}$, and substrate concentration in effluent stream ($S_R$) in g(ml)$^{-1}$.

Fig. 1b. Diagrammatic representation of the maintenance energy fermenter. The symbols are as defined in Fig. 1a.
would correct the formula. The equation in revised and correct form would be:

$$\frac{dx}{dt} = \frac{Y_P A_M (S_R - S_F)}{V_F} - M_G Y_X X$$

where $M_G$ is the maintenance energy coefficient expressed in substrate (g bacterial mass)$^{-1}$(h)$^{-1}$.

In the dialysis culture system, the substrate concentration in the fermentation container ($S_F$) and the glucose concentration in the reservoir container ($S_R$) will depend on the apparatus design and on the microorganism in culture. Mathematically $S_F$ is a function of the substrate affinity of the culture and is defined as:

$$S = \frac{K_S}{\frac{\mu_{\text{max}}}{D}}$$

where $K_S$ is the cell growth constant dependent on cell-substrate affinity in g(ml)$^{-1}$, $\mu_{\text{max}}$ is the maximum specific growth rate constant in (h)$^{-1}$, and $D$ is the dilution rate in (h)$^{-1}$. $S_R$ is a function of membrane permeability and is described as:

$$S_R = \frac{F_M S_F + F_R S_R^0}{F_M + F_R}$$

where $S_R^0$ is the substrate concentration in the reservoir feed in g(ml)$^{-1}$ and $F_R$ is the medium flow rate through the reservoir in ml(h)$^{-1}$.

The relationship described in Equation 11 indicates that as growth continues in the system the maintenance demands
of the population, expressed in the term \( Y_E Y_A X \), will increase and \( \frac{dx}{dt} \) will decrease until \( \frac{dx}{dt} \) goes to zero. At this point, equation 12 will reduce, after rearrangement, to:

\[
X = \frac{Y_E Y_A (S_R - S_F)}{M C V_F}
\]

15

This equation predicts that if \( F_R \) and \( S_R^0 \) are kept constant, the ultimate or maximum cell biomass in this system will be a function of the culture's maintenance demand and substrate affinity and of membrane permeability.

These equations can be modified to describe the maintenance energy fermenter shown in abbreviated form in Figure 1b. In this system, the medium reservoir is not undergoing dialytic transfer and the effluent of the medium reservoir is going directly into the fermentation container. Therefore, the transfer of substrate (\( F_R A_M \)) to the fermentation container becomes \( F_R \); \( S_F \) remains as described in Equation 13 and Equation 15 reduces to:

\[
X = \frac{F_R (S_R^0 - S_F)}{M C V_F}
\]

16

Equation 16 predicts that the mass in the maintenance energy fermenter will be a function of the substrate provision rate (the product of \( F_R \) times \( S_R^0 \)), the fermenter volume, the substrate affinity of the culture and the maintenance energy demands of the culture. The obvious advantage of this system over dialysis culture is that the permeability of the dialysis membrane is not a defining parameter of the system.
Based on the analysis of the dialysis culture by Schultz and Gerhardt (1969), the growth pattern in the maintenance energy fermenter should consist of at least three phases. Cell growth at first should be exponential and would proceed until the substrate concentration in the fermenter became growth-limiting. After exponential growth, growth would be linear and the rate of mass increase would be proportional to the rate of substrate provision. Equation 12 can be modified to describe the linear growth phase in the maintenance energy fermenter, since \( P_{MA} \) is equal to \( F_R \). Equation 12 would become:

\[
\frac{dx}{dt} = Y_X \left[ \frac{F_R(S_R - S_F)}{V_F} - m_G X \right]
\]

This equation predicts that the rate of change of mass will be linear at the outset of substrate dependent growth if the maintenance demands of the population \( m_G X \) are small compared to the rate of incoming substrate \( F_R(S_R - S_F) \). When the maintenance demands become significant in terms of incoming substrate, \( dx/dt \) will decrease and continue to decrease until \( dx/dt \) is equal to zero. Equation 16 will define this state \( (dx/dt = 0) \) where the incoming substrate is just sufficient to provide the maintenance demands of the population. Equation 16 also predicts that the period following substrate dependent growth will be a steady state of constant mass without growth.

F. Characterization of Physiological States in Maintenance Energy Fermentation.

A microbial population growing in the maintenance energy fermenter where its ultimate population density is a function
of its maintenance energy requirements would be ideal for the investigation of the processes that contribute to energy of maintenance and the means by which a cell regulates its growth in response to the amount of substrate available. Since protein turnover is postulated to be one of the primary processes contributing to maintenance energy (Marr et al., 1963; Hempfling and Mainzer, 1975) an investigation into protein turnover in maintenance energy fermenter cultures would indicate how protein turnover contributes to energy of maintenance.

Protein Turnover

Protein degradation serves an important function in bacterial cells. Through protein turnover bacteria can eliminate abnormal and potentially harmful polypeptides which may result from mutations, biosynthetic errors or spontaneous denaturation. Increased protein turnover appears to be an important physiological adaptation to poor nutritional conditions (Goldberg and St. John, 1976).

There is some conflict among various investigators as to the true rate of protein turnover in growing and non-growing bacterial populations. Mandelstam (1957) in an early investigation into protein turnover indicated that the rate of protein turnover in growing cells of E. coli increased from an undetectably low rate of turnover to 5% (h)^-1 when cells were deprived of either a nitrogen source or a carbon source (Mandelstam and Halvorson, 1959). Mandelstam also found that the rate of intracellular protein degradation in non-growing cells was balanced by the rate of reincorporation of the amino
acids into proteins. Other investigators, notably Pine (1966; 1970) and Nath and Koch (1970), found entirely different results. The conflict in these investigations may have resulted from different experimental methodologies used for protein turnover and the measurement of different components of protein turnover.

Protein turnover may be differentiated into two categories; intercellular (the process where amino acid residues derived from damaged or secreting cells are neutralized by other cells) and intracellular (the process where a cell degrades and resynthesizes proteins exclusively within itself) (Levine, 1965). The most commonly measured type of protein turnover is intracellular. Most studies which measure intracellular turnover base the experiments on the rationale: 1) if bacteria are grown from a small population size in the presence of an amino acid label, all species of protein will become labeled; 2) in certain species, i.e. E. coli, the equilibration of intracellular and extracellular pools of amino acids is extremely rapid and the loss of a labeled amino acid from proteins and the entrapment of that label in a large extracellular pool of unlabeled amino acids is a measure of the degradation of the protein (Mandelstam, 1960). This method of determining intracellular protein degradation may be referred to as amino acid exchange. One of the pitfalls in this procedure is that if various classes of proteins turnover at different rates, the protein that is degraded and resynthesized most rapidly will have the highest specific activity and will be reflected in the measurements of protein turnover.
(Marr et al., 1963; Mandelstam, 1957; Koch and Levy, 1957) into protein turnover treated it as a kinetically homogeneous process.

When protein turnover was recognized as a kinetically heterogeneous process, as well as better methodologies developed, several of the conflicts involving protein were resolved. Table 2 lists the rates of protein turnover found by various authors and the methodologies used. The loss of amino acid from intracellular protein was referred to as protein degradation since turnover implies the degradation of proteins to amino acids as well as the reincorporation of the amino acids into protein. Mandelstam (1957) and Koch and Levy (1957) indicated that protein in exponential cells was stable or decayed very slowly. Koch and Levy used an internal trap (degradation of labeled proteins to labeled glycine which as a purine precursor, became trapped in nucleic acids) to measure the rate of degradation \(<0.1\% (h)^{-1}\) in growing cells. In subsequent investigations, where protein was pulse labeled, protein was found to be degraded in growing cells at two distinct rates. Pine (1970) and Nath and Koch (1970) found that intracellular protein was degraded at a rate of 2.5 to 3.0\% (h)^{-1}. Nath and Koch used a perfusion apparatus to wash the extracellular amino acids (degradation products of protein) away from the culture, thereby eliminating errors that may arise from extensive experimental manipulations of the culture. These authors also found a slower, isotope concentration and culture condition independent, protein degradation rate. They
<table>
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<tr>
<th>Strain</th>
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<th>Protein Component Measured</th>
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<tr>
<td>E. coli PS</td>
<td>Glucose-limited</td>
<td>3.2</td>
<td>a</td>
<td>b</td>
<td>Marr, Nilson &amp; Clark, 1963</td>
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<tr>
<td>E. coli B(lep)</td>
<td>Non-growing (amino acid starvation)</td>
<td>5.1</td>
<td>d</td>
<td>e</td>
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<tr>
<td>E. coli B</td>
<td>Exponential stable</td>
<td>0.1</td>
<td>a</td>
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<tr>
<td>E. coli B</td>
<td>Exponential</td>
<td>3.6</td>
<td>d</td>
<td>e</td>
<td>&quot;</td>
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<tr>
<td>E. coli GL(lep)</td>
<td>Non-growing (amino acid starvation)</td>
<td>2.5</td>
<td>g</td>
<td>e</td>
<td>Pine, 1970</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>g</td>
<td>e</td>
<td>&quot;</td>
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<tr>
<td>E. coli B</td>
<td>Exponential (glucose grown)</td>
<td>2.5</td>
<td>g</td>
<td>e</td>
<td>Nath &amp; Koch, 1970</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 - 0.6</td>
<td>g</td>
<td>h</td>
<td>&quot;</td>
</tr>
<tr>
<td>E. coli K12</td>
<td>Exponential</td>
<td>0.16- 0.18</td>
<td>i</td>
<td>j</td>
<td>Levine, 1965</td>
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Table 2. continued

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<td>a.</td>
<td>Average intracellular protein degradation.</td>
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<tr>
<td>b.</td>
<td>Continuous labeling followed by amino acid exchange.</td>
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<tr>
<td>c.</td>
<td>Continuous labeling, internal trap.</td>
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<td>d.</td>
<td>Intracellular protein degradation of newly labeled most active substrates.</td>
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<td>e.</td>
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<td>f.</td>
<td>Intracellular protein degradation of aged most active substrates.</td>
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<td>g.</td>
<td>Intracellular protein degradation of most active substrates.</td>
</tr>
<tr>
<td>h.</td>
<td>Perfusion apparatus, pulse labeling, amino acid exchange.</td>
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<td>i.</td>
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<td>j.</td>
<td>Equilibrium apparatus, continuous labeling, appearance of $^{14}$C-threonine in unlabeled culture.</td>
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</tbody>
</table>
hypothesized that the lower rate may have been a result of cell lysis or sloughing of protein from the cell.

The intercellular component of protein turnover was measured by Levine (1965) in an equilibrium apparatus where culture filtrates were exchanged between $^{14}$C-threonine labeled and unlabeled cultures. The rate of intercellular turnover in a growing culture of *E. coli* was found to be $0.16-0.18\%(h)^{-1}$.

The studies of intracellular protein degradation by Pine (1966) indicated that growing cells catabolize newly synthesized protein at a rate comparable to starving cells but metabolize more stable protein at a rate less than half that of the newly synthesized proteins. Pine further concluded that intracellular protein degradation appears to be a constitutive process similar under conditions of cellular growth and growth arrestment, provided turnover is measured over short time periods and from the proteins most actively degraded. Both Pine (1970) and Nath and Koch (1970) found that in growing cultures the rate of protein degradation was inversely proportional to its age and that cell proteins in older, more slowly growing cells are less susceptible to proteolysis than are more recently synthesized proteins. Since the rate of proteolysis was nearly constant in the slowly growing cell, a fairly proportionate increase in the degradation of proteins of all ages must occur.

The rate of intracellular protein degradation determined for growing cultures does not apply to all proteins within the cell. Nath and Koch (1970) calculated that only
2 to 7% of the total cellular protein is degraded at the rapidly occurring rate.

Protein degradation in *E. coli* is regulated coordinately with net RNA synthesis and other growth-related processes (Goldberg and St. John, 1976). Conditions which reduce either the supply of amino acids for protein synthesis or alter the energy levels of cells enhance proteolysis and decrease RNA synthesis. These changes have been correlated with changes in the intracellular levels of guanosine tetraphosphate (Goldberg and St. John, 1976).

Increased protein degradation in *E. coli* has been demonstrated in conditions when cells are deprived of amino acids, nitrogen, glucose and various inorganic nutrients (phosphate, potassium, and magnesium) (Goldberg and St. John, 1976). *E. coli* also selectively degrades aberrant protein which may be produced by mutational, biosynthetic or posttranslational events. These aberrant proteins which are subject to increased proteolysis rates are of several types: 1) proteins that contain amino acid analogs, 2) prematurely terminated polypeptides, 3) incomplete proteins that contain normal carboxyl ends but abnormal amino terminals, 4) certain proteins that are produced in certain temperature-sensitive or osmotic-remedial mutants, 5) the excess subunits (B and B') of RNA polymerase produced in merodiploids, and 6) proteins that arise from reduced fidelity of protein synthesis (Goldberg and St. John, 1976).

Two distinct degradative systems have been postulated to function in proteolysis in *E. coli*, one in the degradation
of aberrant proteins and one in the degradation of proteins produced during the deprivation of an essential nutrient (Gottesman and Zipser, 1978; Miller and Zipser, 1977). The degradation of aberrant proteins appears to require energy since degradation of this class of proteins is inhibited by energy poisons such as cyanide or azide (Gottesman and Zipser, 1978). In E. coli it is unclear whether the degradation systems involve different proteolytic enzymes (Goldberg and St. John, 1976). There is evidence that serine proteases are involved in the degradation of proteins produced in E. coli which was deprived of glucose. The inhibition of these enzymes did not reduce the rate of proteolysis of analog containing proteins but did inhibit the rate of proteolysis in E. coli deprived of glucose (Goldberg and St. John, 1976).

**β-Galactosidase Induction.**

Investigations concerning the enzyme, β-galactosidase, would be extremely useful in assessing the protein synthesizing capability, turnover of a single type of enzyme and genetic regulation in maintenance energy fermenter cultures of E. coli. Theoretically, this enzyme would be inducible in maintenance energy phase cultures of E. coli B when the glucose concentration in the fermenter falls to its minimum level, which occurs at the end of exponential growth phase. Prior to this time, the enzyme would be subject to catabolite repression due to glucose excess (Pastan and Perlman, 1970). The inducibility of β-galactosidase in chemostat cultures when grown at low
specific growth rates (residual glucose in fermentation vessel essentially zero) has been reported (Dean, 1972).

β-galactosidase has been induced in cultures of *E. coli* which were in various states of energy limitation or depletion of an essential nutrient. The rate of β-galactosidase synthesis in an arginine-requiring strain starved for arginine was comparable to the rate of synthesis in unstarved cells (Mandelstam, 1961). Uracil deprivation did not significantly alter the cell's ability to synthesize β-galactosidase but thymine deficiency reduced the rate of synthesis of β-galactosidase by about 30% (Mandelstam, 1961). Mandelstam (1961) also studied the rate of total protein synthesis in cells induced for β-galactosidase and concluded β-galactosidase forms an approximately constant fraction of the total protein synthesized whether the cells are growing or not. Mandelstam did report that the presence of a carbon source such as succinate, when cells were starved for nitrogen or an amino acid, inhibited β-galactosidase synthesis. Mandelstam explained the capacity for β-galactosidase synthesis in non-growing cells as being a result of protein degradation and incorporation of the freed amino acids into β-galactosidase. The measured rate of protein degradation (5% per h) was comparable to the observed rate of β-galactosidase synthesis. Palmer and Mallette (1961) also found that cells of *E. coli* could synthesize enzyme in the absence of both exogeneous nitrogen and energy.

The rate of β-galactosidase synthesis in exponential phase seems to be tightly coupled to the overall rate of
protein synthesis since a constant proportion of the newly synthesized cell mass is $\beta$-galactosidase (Bruenn and Kane, 1976).

**Bacteriophage Infection**

The response of maintenance energy phase cultures of *E. coli* B to bacteriophage infection should be indicative of the energy state of the cell and whether the maintenance energy phase cell tightly controls the cellular processes which require energy.

Since a bacterial population must be brought to the maintenance energy phase under energy-limiting conditions, bacteriophage infections of maintenance energy phase cells must be studied with a bacteriophage capable of infecting *E. coli* B in a minimal medium. The infectivity of bacteriophage $T_4$ for *E. coli* B grown in minimal medium has been shown to consist of two subpopulations of bacteriophage in terms of their requirement of tryptophan for adsorption (Anderson, 1948a).

The latent period and the number of viral progeny produced during the growth cycle of $T_4$ have been demonstrated to be a function of the host cells' physiological state and the medium in which it is grown. Delbruck (1940) demonstrated that the latent period was increased from 17 min to 30 min when $T_4$ infected stationary phase cells as opposed to exponential phase cells of *E. coli* B. Concomitantly the burst size was decreased from 170 to 20 phage progeny per infected bacterial host. The latent period has also been demonstrated
to be a function of the growth rate of the host bacteria (Ellis and Delbruck, 1939). The validity of this finding may be questioned since the authors used temperature as a means of altering growth rate and temperature was shown to influence the rate of infection. Growth of T₄ on E. coli grown in minimal medium also reduced the burst size to 30 to 40 viral progeny.

The adsorption of T₄ to the E. coli cell surface involves the specific attachment of tail fibers to the bacterial receptor (Simon and Anderson, 1967a) followed by changes in their contractile tail sheaths and base plates (Simon and Anderson, 1967b). The proposed bacterial receptor site for T₄ is the terminal α-(1 → 3-linked) glucose residue of the lipopolysaccharide molecule (Prehm et al., 1976). Prehm et al. (1967) postulated that the lipopolysaccharide molecule contained receptors for both the long and short tail fibers of T₄. The actual site of adsorption and injection of viral DNA appears to be areas of wall-membrane association (Bayer, 1968). The number of wall-membrane associations found in exponentially growing E. coli was 200 to 400 and this number was matched by the saturation capacity of E. coli by T₄ (Bayer, 1968).
CHAPTER II

MATERIALS AND METHODS

A. The Maintenance Energy Fermenter.

The maintenance energy fermenter is shown schematically in Figure 2. The apparatus was essentially a continuous culture device fitted with a system for biomass feedback. The fermentation vessel consisted of a 1 L, 5-neck boiling flask. Each neck was fitted with a 24/40 teflon adaptor clamped in place.

Culture medium was pumped from the medium reservoir to the fermenter by means of a peristaltic pump (Harvard, model 1201, Millis, MA). Tubing in the medium addition system was either glass or siliconized stainless steel. Silicone rubber tubing was used in the peristaltic pump which was housed in a closed container.

The fermentation vessel and peristaltic pump container were continuously flushed, and the medium reservoir was sparged with oxygen-free nitrogen. Nitrogen was made oxygen-free by passage through an alkaline solution of 10\% (w/v) pyrogallol and 10\% (w/v) Na$_2$CO$_3$. The nitrogen flow rate into the medium reservoir was between 1.0 and 1.5 L(min)$_{-1}$ and the flow rate into the fermentation vessel was between 0.3 and 0.5 L(min)$_{-1}$.

The nitrogen entering the medium reservoir and fermentation vessel was sterilized by passage through a sterile
Fig. 2. Schematic diagram of maintenance energy fermenter showing the defining parameters: fermenter volume ($V_F$) in ml, substrate concentration in medium reservoir ($S_R$) in $\mu$mol/ml$^{-1}$, substrate concentration in fermenter ($S_F$) in $\mu$mol/ml$^{-1}$, cell concentration ($X$) in $\mu$g/ml$^{-1}$, flow rate into fermenter ($F_{in}$) in ml(h)$^{-1}$, and flow rate from separator assembly ($F_{out}$) in ml(h)$^{-1}$.
Fig. 2
filter housing (Balston, model 95P, Andover, MA) containing a sterile microfiber filter tube (model AA). Gas from the fermenter was vented through a sterile filter (Balston, model 90).

The biomass feedback system consisted of a thin channel recirculating separator (Amicon, model TCI R, Lexington, MA), a rotary vane pump (Procon, model 1 621XL, Murfreesboro, TE), coupled to a variable speed motor, and a micrometering valve (Whitney, model SS-22RS4). The separator assembly was fitted with a 0.2 um polycarbonate membrane filter (Nuclepore). The seal and integrity of the membrane filter in the separator were assured by conducting a bubble point test according to the manufacturer's instructions.

The maintenance energy fermenter was operated by pumping culture from the fermenter vessel across the filter and returning the culture to the fermenter. The culture provided to the separator assembly was controlled by the variable speed pump and was usually 220 to 300 ml(min)^{-1}. The rate of filtrate removal (F_S) from the separator assembly was controlled by a micrometering valve and was set equal to the rate of glucose provision (F_R). When F_S was equal to F_R the volume of the fermenter (V_F) remained constant.

The hydrogen ion concentration was monitored with a combination pH electrode fitted directly into the fermentation vessel through one of the necks. The pH of the culture was maintained at 7.0 by the continuous addition of 2N NaOH by a metering pump. The anaerobic nature of the culture was confirmed by monitoring the redox potential of the filtrate with
a combination platinum electrode. The platinum electrode was fitted into a glass container connected to the filtrate line.

The temperature of the culture was maintained in all experiments at $30^\circ \pm 0.5^\circ C$ by placing the fermentation vessel in a constant temperature water bath.

The entire system, except the medium reservoir, was sterilized with a solution of 5% (v/v) formaldehyde for a contact time of 48 h. The formaldehyde was removed by successive rinsing with 3 volumes of sterile medium.

The fermenter was inoculated or sampled through a 3-way ball valve (Whitey, model SS4X86) fitted in the culture return line from the separator. Samples were withdrawn or the inoculum added with a syringe through one port of the 3-way valve which was fitted with a serum bottle cap.

When the apparatus was operated as a chemostat, the separator was removed and the exit line of the fermentation vessel was connected to one channel of the Harvard pump.

B. Culture Medium and Inoculation of Fermenter.

Minimal medium (Davis and Mingioli, 1950) was used in all experiments involving either the chemostat or the maintenance energy fermenter. Glucose at the concentrations ranging from 14.5 to 3.4 $\mu$mol(ml)$^{-1}$ was found to be growth limiting. The reducing agent dithiothreitol (DTT)(Sigma) was added at a concentration of 10 $\mu$g(ml)$^{-1}$ to insure the anaerobic nature of the growth medium. The culture medium was autoclaved in either 10 L or 18 L batches.
Cultures of *E. coli* B were maintained on slants of minimal medium containing 1.25 mg/ml glucose, stored at 4°C and transferred monthly. At 6 month intervals, this culture was replaced by transfer of a culture from -70°C storage. Frozen cultures were maintained in tryptic soy broth supplemented with 0.3% yeast extract, 0.1% glucose and 10% dimethyl sulfoxide.

The fermenter was inoculated with 30 ml of a 10 h culture of anaerobically grown *E. coli* B. The inoculum was grown in minimal medium without DTT and was prepared from cells which had previously been transferred twice under anaerobic conditions. All manipulations of the inoculum were conducted in an anaerobic chamber. All media used in the preparation of the inoculum were reduced prior to use for 24 to 48 h in the anaerobic chamber.

C. **Cellular Assays.**

At regular intervals, samples were withdrawn from the fermenter and analyzed. Each sample was checked for culture purity. If the fermenter was contaminated, it was disassembled, resterilized and the experiment started again. Cell dry weights were determined in triplicate by filtering portions of the sample onto pre-weighed polycarbonate membrane filters (25 mm diameter, 0.2 μm, Nuclepore). The filter was rinsed with 1 ml of prefiltered 0.85% (v/v) formaldehyde. Each filter was brought to constant weight at 60°C and weighed with a Cahn balance. Bacterial numbers were determined in duplicate by the spread plate method. The diluent was prewarmed (30°C)
minimal medium containing 1.25 mg/ml\(^{-1}\) glucose. The plates were incubated at 30\(^\circ\)C for 48 h and then counted. Culture turbidity was monitored on a Klett-Summerson instrument fitted with a number 54 filter.

Cellular DNA was determined by fluorescence using ethidium bromide (Donkersloot, Robrish and Krichevesky, 1972). The DNA samples were prepared by filtration onto 0.2 \(\mu\)m polycarbonate membrane filters and were rinsed with 1 ml of a saline-EDTA solution. The filtered cells were resuspended in 2 ml of 0.3 N KOH, hydrolyzed at 37\(^\circ\)C and then neutralized with 0.5 ml of 1 N HCL. The samples were then frozen for analysis at a later date. When assayed, an equal volume of a 4 \(\mu\)g/ml\(^{-1}\) solution of ethidium bromide was added and the fluorescence measured in a Turner 111 fluorometer. Calf thymus DNA (Sigma) was used as a standard.

Cellular RNA was determined by the orcinol procedure. Cells were frozen for analysis at a later date in 0.8% formaldehyde. After thawing, the sample was washed three times with cold trichloracetic acid (TCA), heated in 5% TCA for 15 min and then assayed. Yeast RNA (Sigma) was used as the standard.

Protein was assayed by a modification of the Lowry technique (Brunschede, Dove and Bremer, 1977). Cells were hydrolyzed by diluting (1:5) into 0.5 N NaOH and boiling for 5 min. The samples were frozen for analysis at a later date. Bovine serum albumin fraction V (Fisher) was used as the standard.
D. Assay of Culture Viability.

Culture viability was determined by a modification of the microcolony technique (Postgate et al., 1961). The technique of Postgate was adapted for use with membrane filters. Samples taken from the fermenter were immediately diluted into prewarmed (30°C) and prefiltered minimal broth. One ml of the appropriate dilution was filtered onto a 0.2 μm cellulose acetate, 25 mm diameter filter (Millipore, type HA) so that when observed microscopically with an oil immersion objective, 20-30 bacteria were seen per field. Immediately after filtration the filters were placed in petri dishes which contained either tryptcase soy agar supplemented with 0.3% yeast extract and 0.1% glucose or minimal agar containing 1.25 mg/ml-1 glucose. Both media contained 1.4% agar instead of the usual 1.5%. The plates were inverted and incubated at 37°C. The tryptcase soy agar was incubated for 3-4 h and the minimal agar for 6-8 h. After incubation, the filters were removed and dried at 60°C for 20 min. The filters, when made transparent with immersion oil, were observed by phase-contrast microscopy at a magnification of 970x.

The criterion used to differentiate viable and non-viable cells was that a viable cell would undergo cell division and give rise to a microcolony, whereas a nonviable cell would not. A minimum combined total of 300 single cells and microcolonies was routinely counted. The accuracy of the technique was checked by using a mixture of heat killed and exponential phase cells of E. coli B and determining the percent viability.
E. Determination of Filtrate Components.

The presence of DTT in the filtrate was found to interfere with the analysis of RNA, DNA, protein and glucose. Each assay was corrected for this interference. The salt concentration in filtrate samples used in the analysis of DNA, RNA, protein and amino acids was reduced by passage through a column containing Amberlite, IR-120 (H+) (Fisher). Each sample was passed through the column twice and the column washed with a volume of distilled water equal to the bed volume. Amino acids were eluted with 0.5 N NH₄OH and taken to dryness.

Protein in the filtrate was determined on the treated samples using the Lowry procedure (Lowry et al., 1951). RNA was determined by the orcinol reaction. DNA was determined by the diphenylamine technique (Burton, 1956). Amino acid concentrations in the filtrate were determined on a Beckman Automated Amino Acid Analyzer.

The glucose concentration of filtrate was determined by using a commercial glucose oxidase kit (Worthington Biochemical Corp.). The presence of DTT was corrected for by using N-ethyl maleimide (Eastman) in the procedure (Davies and Wayman, 1973).

Total carbohydrate concentration in the filtrate was measured by the phenol-sulfuric acid assay (DuBuis et al., 1956).

F. Lipopolysaccharide Analysis.

Cellular and extracellular lipopolysaccharide (LPS) was determined by the Limulus lysate procedure. All glassware
and dry chemicals used in the preparation of and assay for LPS were made pyrogen-free by heating at 180°C for 3 h. Pyrogen-free water was prepared by collecting water from a Milli-Q water purification system (Millipore) in pyrogen-free containers and autoclaving at 121°C for 3 h. The lysate was prepared by the method of Jorgenson and Smith (1973) and modified as recommended by Sullivan and Watson (1974). The modification consisted of the addition of pyrogen-free CaCl_2 to the lysate such that the final concentration of the CaCl_2 was 0.02 M. The assay procedure was that of Jorgensen and Smith (1973) with pyrogen-free 0.02 M CaCl_2 as the diluent. The endpoint of the lysate was determined with standard LPS (Sigma, phenol extract of *E. coli* serotype O111:B4).

Cellular LPS was determined from fermenter samples that had been centrifuged at 9,000 Xg for 10 min and resuspended in pyrogen-free distilled water. Extracellular LPS was determined on filtrate samples collected at the same time as the samples for the determination of cellular LPS.

G. Assay for Fermentation Products.

The concentration of fermentation products in the filtrate was determined by gas-liquid chromatography using a Perkin Elmer model 3920 gas chromatograph equipped with a hydrogen flame detector. The fermentation products were separated on a stainless steel column (0.32 by 305 cm) packed with 10% SP-1000 (1% H_3PO_4) on 100/120 mesh Chromosorb WAW (Supelco). Chromatographic conditions used in the analysis were: 1) carrier gas (N_2) flow rate, 40 ml(min)^{-1}; 2) injection port temperature 205°C; 3) detector temperature 230°C;
and 4) column temperature of 50°C, 120°C and 160°C for the analysis of ethanol, lactic and succinic acids, and acetic acid, respectively.

Samples and standards were prepared for injection by the procedure described in the Anaerobe Laboratory Manual (Holdeman and Moore, 1975). A sample size of 1 μl was routinely injected.

H. Radioisotope Experiments.

Incorporation of substrate carbon and loss of cell-bound carbon was determined by addition of 0.2 mmol of D-[^14]C glucose [2.3 mCi/(mmol)] (New England Nuclear) directly to the fermentation vessel. Twenty minutes after the addition of labeled glucose and at regular intervals thereafter, 1 ml samples were withdrawn and diluted 1:10 into 0.8% formaldehyde. Cells were collected on a 0.2 μm polycarbonate membrane (Nuclepore) and washed with 1 ml of 0.8% formaldehyde. The filters were placed into scintillation vials and dissolved by the addition of 1 ml of Protosol (New England Nuclear). After the filters were dissolved, 50 μl concentrated acetic acid, followed by 10 ml of a xylene-based scintillation solution were added. The radioactivity was determined at each sampling time on duplicate samples using a Packard Tri-carb Scintillation Spectrophotometer.

The loss of cellular DNA and protein was determined by labeling the culture with 14C-leucine and 3H-thymidine and following the disappearance of label from the cells. The radionuclides were added directly to the fermentation vessel
as a mixture of the radionuclides and their respective unlabeled compounds. The mixture contained 0.041 μmol \(^{3}H\) thymidine \(6.7 \text{ Ci(mmols)}^{-1}\) (New England Nuclear), 3 μmol unlabeled thymidine (Cal Biochem), 3.2 μmol \(^{14}C\) leucine \(287 \text{ mCi(mmols)}^{-1}\) (New England Nuclear), and 7.0 μmol unlabeled L-leucine (Calbiochem). Samples were prepared and counted as outlined above.

I. Induction of the Lactose Operon and Assay of β-galactosidase Activity.

Cultures were induced for β-galactosidase by the addition of the inducer directly to the fermentation vessel. Either the gratuitous inducer isopropyl-β-D-thiogalactopyranoside (IPTG), or the natural inducer, lactose, were used. The inducer was always added so that the final concentration in the fermenter vessel was \(10^{-3}\) M. When the culture was to be continuously exposed to the IPTG, the inducer was added to both the fermentation vessel and medium reservoir. In all other experiments, the inducer was added only to the fermentation vessel.

β-galactosidase activity was determined on samples withdrawn from the fermenter with ortho-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. The assay procedure outlined by Miller (1972) was followed. The cells were disrupted with sodium dodecyl sulfate and chloroform. The amount of enzyme activity determined in the assay was corrected for culture turbidity. β-galactosidase activity was determined on duplicate samples. Accepted values were within 5%.
An enzyme unit was defined as the amount of enzyme that will hydrolyze $10^{-9}$ mol(min)$^{-1}$ of ONPG at 28°C.

**J. Virus Infection and Analysis of Viral Numbers.**

The response of a maintenance energy phase culture of *E. coli* B to infection by bacteriophage T4 was determined by adding the phage to the fermenter in phase 3 and assaying for viral numbers. Virus for addition to the fermenter was prepared in aerobic cultures of *E. coli* B grown in minimal medium. The lysate was centrifuged at 10,000 X g for 15 min and the supernatant fluid was decanted and stored at 4°C until used.

Virus assays of the infected maintenance energy phase culture consisted of determining the number of plaque-forming units in four fractions of the sample. Samples withdrawn from the fermenter were centrifuged at 10,000 X g for 10 min. The supernatant fluid was decanted and assayed for viral numbers. The number of virus occurring in this fraction represented the free or unattached virus in the infected culture. The pellet was resuspended to the original volume in minimal medium and divided into three fractions. One, fraction, without modification, was assayed for the number of viral particles. This fraction represented cell-associated virus. Another fraction was treated with chloroform and assayed for virus. This fraction represented intact virus. The remaining fraction was blended in a Sorvall microhomogenizer cup at full speed for 1 min and assayed for virus. This fraction represented reversibly-attached virus.
Viral numbers were determined by the soft agar overlay procedure. This assay consisted of pipetting 0.1 ml of a virus dilution made in minimal broth onto a petri dish containing nutrient agar and overlaying the virus suspension with molten agar containing 0.8% agar and approximately $5 \times 10^7$ E. coli B (ml)$^{-1}$. The virus suspension was dispersed into the soft agar overlay and the agar was allowed to solidify. The petri dishes were inverted, incubated at 37°C for 24 h and the number of plaques counted.

K. Growth Inhibition by Penicillin Addition.

The response of a maintenance energy phase culture to a growth inhibiting antibiotic was determined by the addition of penicillin to the culture. Penicillin G (Lilly) was added to the fermentation vessel and medium reservoir at a final concentration of 200 units (ml)$^{-1}$. Penicillin at this concentration was found to completely inhibit growth of anaerobic cultures of E. coli B.

L. Electron Microscopy.

Samples for thin sectioning were withdrawn from the fermenter and fixed by the addition of one volume of 0.1% $\text{OsO}_4$ in Kellenberger buffer (Kellenberger, Ryter and Sechaud, 1958). The cells were washed once with Kellenberger buffer and suspended in two drops of tryptone salts solution. The resuspended cells were brought to 47°C in a waterbath and 2% Nobel agar (Difco) was added. The agar-cell suspension was placed on a glass slide, allowed to solidify and cut into small cubes. The samples were post-fixed in 1% $\text{OsO}_4$ in
Kellenberger buffer overnight at room temperature, followed by staining in 0.5% uranyl acetate in Kellenberger buffer for 2-3 h. The samples were dehydrated in gradations of ethanol and embedded in Spurr's low viscosity resin (Spurr, 1969). The thin sections were stained in 0.5% uranyl acetate in methanol for 15 to 60 min, followed by 0.4% lead citrate for 1-5 min (Reynolds, 1963), and were photographed in a Phillips EM-200 electron microscope operating at 60 kv.

Negative stains were made by adding cell samples to parlodian carbon-coated 200-mesh copper grids. The grids were stained in 1% phosphotungstic acid for 1 min and then photographed.
CHAPTER III

RESULTS

The maximum obtainable biomass in a culture system such as shown in Figure 1, where all biomass produced as a result of the metabolism of incoming nutrient is retained within the system, should be determined by the populations' maintenance energy requirements. The ultimate mass in this system should be predicted by equation 16. A correction should be applied to equation 16 to account for the amount of glucose carbon that is incorporated into cell biomass and therefore unavailable for use as maintenance energy. The modification of equation 16 yields:

$$X_{\text{max}} = \frac{V_F (S_R - S_F - S_A)}{V_F m_c}$$

where $X_{\text{max}}$ is the maximum cell concentration in mg(ml)$^{-1}$, $V_F$ is the fermenter volume (450 ml), $S_A$ is the amount of glucose incorporated into cell carbon in $\mu$mol(ml)$^{-1}$, $S_F$ is the concentration of glucose in the fermenter in $\mu$mol(ml)$^{-1}$, $S_R$ is the concentration of glucose in the reservoir in $\mu$mol(ml)$^{-1}$, and $m_c$ is expressed in umol glucose (mg bacterial dry weight)$^{-1}$(h)$^{-1}$.

Equation 18 predicts that the ultimate population size in the maintenance energy fermenter will be determined
by the cell's maintenance energy requirements, the residual glucose in the fermenter \((F_R S_F)\), the amount of substrate incorporated into biomass \((F_R S_A)\), and the amount of glucose provided \((F_R S_R)\) to the fermenter. The amount of glucose provided to the fermenter is termed the glucose provision rate \((GPR)\) and is expressed in \(\mu\text{mol glucose(h)}^{-1}\).

A. Determination of \(m_c\) and \(Y_{\text{max}}\).

The apparatus shown in Figure 2 was modified and used as a chemostat to obtain the value of the maintenance coefficient for use in predicting \(X_{\text{max}}\) when the apparatus was used as a maintenance energy fermenter. The maintenance coefficient of a culture growing in the chemostat can be calculated from the formula (Firt, 1965):

\[
\frac{1}{Y_a} = \frac{m_c}{\mu} + \frac{1}{Y_{\text{max}}}
\]

where \(Y_a\) is the apparent growth yield in mg cell dry weight (\(\mu\text{mol glucose consumed})^{-1}\), \(\mu\) is the specific growth rate, and \(Y_{\text{max}}\) is the maximum growth yield and is expressed in the same units as \(Y_a\). This equation was solved graphically by plotting the data of Table 3 in the form shown in Figure 3. A small correction for the assimilation of glucose into cell-bound carbon was applied to the data used to calculate \(Y_a\) on the basis that carbon comprises 45.6\% of the bacterial dry weight in \textit{Escherichia coli} B (Hempfling and Mainzer, 1975). The amount of glucose metabolized was calculated by using the relationship:
Table 3. Yield Characteristics of *Escherichia coli* B Growing
in Glucose-Limited Anaerobic Continuous Culture.\(^a\)

<table>
<thead>
<tr>
<th>( \frac{D}{\text{hr}^{-1}} )</th>
<th>Dry wt ( \frac{\mu g (\text{ml})^{-1}}{\text{hr}} )</th>
<th>Residual glucose ( \frac{\mu mol (\text{ml})^{-1}}{\text{hr}} )</th>
<th>Glucose metabolized ( \frac{\mu mol (\text{ml})^{-1}}{\text{hr}} )</th>
<th>( Y^c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125</td>
<td>294</td>
<td>0.007</td>
<td>12.6</td>
<td>23.4</td>
</tr>
<tr>
<td>0.148</td>
<td>306</td>
<td>0.013</td>
<td>12.5</td>
<td>24.5</td>
</tr>
<tr>
<td>0.235</td>
<td>324</td>
<td>0.007</td>
<td>12.4</td>
<td>26.1</td>
</tr>
<tr>
<td>0.420</td>
<td>159</td>
<td>7.510</td>
<td>5.94</td>
<td>26.8</td>
</tr>
</tbody>
</table>

\(^a\)Conditions: 340 ml vessel, 14.5 \( \mu \text{moles (ml)}^{-1} \) glucose in medium reservoir.

\(^b\)Glucose metabolized = glucose concentration in reservoir minus glucose concentration in effluent (residual glucose) minus glucose equivalent of incorporated carbon (Hempfling and Mainzer, 1975).

\(^c\)\( \mu g \) cell dry wt(\( \mu \text{mol glucose metabolized} \))\(^{-1} \).
Fig. 3. Relationship between $Y_e$ and $\mu$ for an anaerobic chemostat culture of *Escherichia coli* B.
\[ S = S_R - S_F - S_C \]

where \( S \) is the amount of substrate metabolized \( \mu \text{mol} (\text{ml})^{-1} \), \( S_R \) is the glucose concentration in the medium reservoir \( \mu \text{mol} (\text{ml})^{-1} \), \( S_F \) is the residual glucose concentration in the fermentation vessel \( \mu \text{mol} (\text{ml})^{-1} \) and \( S_C \) is the glucose equivalent of incorporated carbon.

The \( m_c \) value as determined from the slope of the line in Figure 3 is 0.00096 \( \mu \text{mol glucose (\mu g bacterial dry wt)}^{-1} (h)^{-1} \). The \( Y_{\text{max}} \) value as determined from the \( Y \) intercept of Figure 3 is 28.9 \( \mu \text{g bacterial dry wt(\mu mol glucose)}^{-1} \).

**B. Description of the Growth Phases of Maintenance Energy Cultivation.**

*Escherichia coli* B grown in the maintenance energy fermenter displayed the growth pattern shown in Figure 4. The pattern of growth shown in Figure 4 has been confirmed in 22 separate experiments. Growth in the system, as shown by the change in cell mass followed three sequential phases: exponential growth, substrate dependent growth and the maintenance phase. Exponential growth (Phase 1) proceeded from the time of inoculation to a point approximately 8 - 10 hours later. At this time, an analysis of glucose in the filtrate indicated that glucose was present in a concentration below 1 \( \mu \text{g(ml)}^{-1} \). A period of substrate-dependent growth in which bacterial dry weight increased as a linear function of time followed phase 1 and proceeded for an additional 15 hours. At approximately 25 hours after inoculation, the growth rate
Fig. 4. The change in bacterial mass (○) and numbers (■) during the three phases of maintenance energy cultivation. GPR was 387 μmol(h)^{-1} at an $V_R$ of 56 ml(h)^{-1}.
decreased from 19 ug bacterial dry wt (ml)^{-1}(h)^{-1} in phase 2 to 9 ug bacterial dry wt (ml)^{-1}(h)^{-1}. This third phase of reduced growth rate is termed the maintenance energy phase (Phase 3). Bacterial numbers followed closely the three phase pattern displayed by bacterial mass except that cell numbers reached their phase 3 rate of increase approximately 3 hours before bacterial dry weight exhibited the phase 3 growth rate. An analysis of population viability by the microcolony technique indicated that the population was more than 92% viable throughout phases 2 and 3.

The changes in cellular macromolecules during the 3 phases are shown in Figure 5. During phases 2 and 3 DNA, RNA and protein followed the same growth general pattern as did bacterial dry weight. The point at which the rate of change of DNA, RNA and protein decreased from the phase 2 rate to the phase 3 rate occurred at the same time and was identical to the point at which the bacterial dry weight rate changed.

The rate of change of various culture parameters is shown in Table 4. These data indicate that both phase 2 and phase 3 are periods of unbalanced growth with DNA, RNA, protein, bacterial dry weight and bacterial numbers increasing at different rates. During phase 2, bacterial numbers increased faster than dry weight and the cells were becoming reduced in percent composition of RNA and enriched in protein and DNA. Phase 3 growth consisted of cells becoming enriched in protein and reduced in percent composition of RNA and DNA. The rate of change of cell numbers in relation to bacterial dry weight indicated that changes in cellular geometry were
Fig. 5. Change in bacterial mass (○), cell numbers (■), DNA (▲), RNA (□), and protein (▲) in phase 2 and phase 3. GPR was 416 \(\mu\text{mol(h)}^{-1}\) at an \(F_R\) of 60 ml(h).
Table 4. Rate of Change of Culture Parameters in Phase 2 and Phase 3.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Percent Increase in Parameter Per Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phase 2</td>
</tr>
<tr>
<td>DNA</td>
<td>7.9</td>
</tr>
<tr>
<td>RNA</td>
<td>3.3</td>
</tr>
<tr>
<td>Protein</td>
<td>5.0</td>
</tr>
<tr>
<td>Dry Weight</td>
<td>4.5</td>
</tr>
<tr>
<td>Cell Numbers</td>
<td>7.0</td>
</tr>
</tbody>
</table>
occurring in phases 2 and 3. This observation is further supported by cell size measurements (Table 5). Both cell size and volume increased as cell growth occurred in phase 3. Cell size was determined by measurements taken from electron micrographs of negatively stained cells. Cell volumes were calculated from the formula:

\[
\text{Volume} = \left[ \frac{4}{3} \pi \left(\frac{W}{2}\right)^3 \right] + \left[ (1-W) \left(\pi \left(\frac{w}{2}\right)^2 \right) \right]
\]

where \( W \) is the cell width in \( \mu m \) and \( l \) is the cell length in \( \mu m \).

The determination of the percent dividing cells from electron micrographs of negatively stained cells (Table 5) indicated that 12.6% of the cells were dividing in late phase 2 and early to mid phase 3. This percentage was lower than for exponential growth. As the cells progressed into phase 3, the percent dividing decreased from 12.6% in early phase 3 to 9.2% in late phase 3.

The lipopolysaccharide (LPS) content of cells growing in phase 1, 2 or 3 remained a constant fraction of bacterial mass (Table 6). Extracellular LPS was proportional to bacterial mass in phase 1 but not in phases 2 and 3. Extracellular LPS concentration decreased in phase 3 to a value of one-half the value occurring in phase 2. Once the extracellular LPS concentration decreased, it remained at this value throughout phase 3. A constant value in phase 3 indicated that extracellular LPS was being produced at a constant rate which was independent of change in bacterial mass.

The ultrastructure of cells obtained from the different growth phases of maintenance energy cultivation is shown in
Table 5. Distribution of Cell Size, Cell Volume and Percent Dividing Cells in a Culture of *Escherichia coli* B Grown in the Maintenance Fermenter.\(^a\)

<table>
<thead>
<tr>
<th>Phase</th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th>Cell Volume (μm³)</th>
<th>Percent Dividing Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>n*</td>
<td>SD c</td>
<td>mean</td>
</tr>
<tr>
<td>Phase 1</td>
<td>1.56</td>
<td>100</td>
<td>0.43</td>
<td>0.42</td>
</tr>
<tr>
<td>Late Phase 2(^d)</td>
<td>1.06</td>
<td>100</td>
<td>0.21</td>
<td>0.63</td>
</tr>
<tr>
<td>Early Phase 3(^e)</td>
<td>nd(^f)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Middle Phase 3(^g)</td>
<td>0.99</td>
<td>50</td>
<td>0.21</td>
<td>0.60</td>
</tr>
<tr>
<td>Late Phase 3(^h)</td>
<td>1.19</td>
<td>100</td>
<td>0.26</td>
<td>0.73</td>
</tr>
</tbody>
</table>

\(^a\) Determinations made from negatively stained cells of a run conducted at a GPR of 387 μmoles (h)\(^{-1}\).

\(^b\) n = sample size.

\(^c\) SD = standard deviation.

\(^d\) 13 hours into phase 2.

\(^e\) 7 hours into phase 3.

\(^f\) nd = not determined.

\(^g\) 24 hours into phase 3.

\(^h\) 48 hours into phase 3.
Table 6. Cellular and Extracellular Lipopolysaccharide Content of *Escherichia coli* B Cultures Grown in the Maintenance Energy Fermenter.

<table>
<thead>
<tr>
<th>Post inoculation time (h)</th>
<th>Phase</th>
<th>Cellular Lipopolysaccharide $\mu g (ml)^{-1}$</th>
<th>Extracellular Lipopolysaccharide $\mu g (ml)^{-1}$</th>
<th>Bacterial Dry wt $\mu g (ml)^{-1}$</th>
<th>Percent LPS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5</td>
<td>1</td>
<td>1.0</td>
<td>0.21</td>
<td>90</td>
<td>1.1</td>
</tr>
<tr>
<td>12.5</td>
<td>2</td>
<td>2.1</td>
<td>0.52</td>
<td>240</td>
<td>0.9</td>
</tr>
<tr>
<td>30.0</td>
<td>2</td>
<td>4.2</td>
<td>0.52</td>
<td>550</td>
<td>0.8</td>
</tr>
<tr>
<td>36.5</td>
<td>3</td>
<td>4.2</td>
<td>0.21</td>
<td>630</td>
<td>0.7</td>
</tr>
<tr>
<td>59.0</td>
<td>3</td>
<td>8.3</td>
<td>0.21</td>
<td>830</td>
<td>1.0</td>
</tr>
<tr>
<td>73.0</td>
<td>3</td>
<td>8.3</td>
<td>0.21</td>
<td>950</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent cellular lipopolysaccharide (LPS) = cellular LPS/bacterial dry wt.
Figure 6-9. An ultrastructural comparison of exponential phase cells (Figure 6) and cells 6 hours into phase 2 (Figure 7) shows phase 2 cells to be smaller in cell length but similar to exponential phase cells. By 10 hours into phase 2, which lasted 14 hours in this run, cells have become plasmolyzed (Figure 8). Other than plasmolysis, no obvious ultrastructural characteristic distinguishes exponential and phase 2 cells. Maintenance energy phase cells (Figure 9) are not significantly different from phase 2 cells. Possibly, the degree of plasmolysis has decreased. The presence of dividing cells in electron micrographs indicated cell division and growth occurred in phases 2 and 3.

C. Analysis of Extracellular Products in the Filtrate of the Maintenance Energy Fermenter.

The appearance of extracellular products in the filtrate may be considered to be a result of the secretion of the product by the cell. No evidence of significant cellular lysis was found. The absence of lactate dehydrogenase (data not presented) in the filtrate was taken as an indication that cell lysis did not occur during the course of an experiment.

The pattern of cellular secretion products appearing in the filtrate is shown in Figures 10 and 11. The data points represent the accumulated amount of secreted product that had been produced up to that sampling time. The following formula relates the concentration of a secreted product in the filtrate to the amount of secretion during a given time interval:
Fig. 6. Early exponential phase cells of maintenance energy fermenter grown culture of *Escherichia coli* B.
Fig. 7. Maintenance energy fermenter grown cells of *Escherichia coli* B which are 6 h into phase 2.
Fig. 8. Maintenance energy fermenter grown cells of *Escherichia coli* B which are 10 h into phase 2.
Fig. 9. Maintenance energy fermenter grown cells of *Escherichia coli* B which are 39 h into phase 3.
Fig. 10. Secretion of DNA (▲), RNA (□), protein (▲) and carbohydrate (●) by a culture of Escherichia coli B. Each datum point represents the total accumulated secretion of a product at that time. GPR was 162 umol(h)^{-1} at an F_R of 23 ml(h)^{-1}.
Figure 10

Phase 2

Phase 3

Amount of product secreted (mg)

Post inoculation time (h)

10 20 30 40 50 60 70 80
Fig. 11. Secretion of DNA (△), RNA (□), protein (▲), and carbohydrate (●) by a culture of *Escherichia coli* B. Each datum point represents the total accumulated secretion of a product at that time. GPR was 416 μmol(h)^{-1} at an F_R of 60 ml(h)^{-1}. 
where $P_{S_1}$ is the amount of secreted product in the time interval $T_0$ to $T_1$ in mg(h)$^{-1}$, $P_{T_1}$ is the concentration of secreted product in the filtrate at $T_1$ in mg(ml)$^{-1}$, $P_{T_0}$ is the concentration of secreted product in the filtrate at $T_0$ in mg(ml)$^{-1}$ and $\Delta t$ is the time interval between $T_1$ and $T_0$ in h.

The accumulated amount of secreted product at any time $t$ was calculated by summing the values of $P_{S_1}$ obtained from the analyses from the time of inoculation to time $t$. Secretion was represented as the total amount of secreted product rather than as a concentration. The concentration of a secreted product in the fermenter is a function of both the rate of secretion and the dilution rate of the product in the fermenter, whereas the amount of secreted product as calculated by the above formula approximates the true rate of secretion.

A comparison of the cellular concentration of DNA, RNA and protein (Figure 5) to the corresponding amounts in the filtrate (Figure 11) indicated that the rates of secretion of DNA, RNA and protein were independent of growth phase. The rates of DNA, RNA and carbohydrate secretion were constant in phases 2 and 3, whereas the rate of protein secretion increased late in phase 3. The amounts of extracellular DNA, RNA and protein were found to be proportional to the glucose provision rate. In an experiment where the glucose provision rate was 2.6 times that of another, the quantity of DNA, RNA and protein increased on the average 2.7 times. This relationship is further defined in Figure 12 where the secretion rate in
Fig. 12. Relationship between DNA (Δ), RNA (○), and protein (▲) secretion in phase 3 and glucose provision rate.
Fig. 12

Product secretion rate (mg(h)^{-1}) vs. Glucose provision rate (umol(h)^{-1})

Graph a:
- Product secretion rate increases linearly with glucose provision rate.
- A linear regression line is drawn through the data points.

Graph b:
- Product secretion rate increases linearly with glucose provision rate.
- A linear regression line is drawn through the data points.

Graph c:
- Product secretion rate increases linearly with glucose provision rate.
- A linear regression line is drawn through the data points.

Graph d:
- Product secretion rate increases nonlinearly with glucose provision rate.
- A nonlinear regression line is drawn through the data points.
phase 3 is shown as a function of the glucose provision rate. The rate of protein secretion (Figure 12c) followed a more complex function of glucose provision rate than either DNA or RNA and was found to be curvilinear in nature. Since free amino acids were not detected in the filtrate, amino acids were lost in the polymeric form.

D. Energy Metabolism in Maintenance Energy Cultivation.

The results of an analysis of the fermentation products produced by E. coli B during the fermentation of glucose in the maintenance energy fermenter are shown in Table 7. The product ratio for ethanol:acetic acid:succinic acid remained approximately constant throughout the 3 phases in the two runs in which the substrate provision rates differed by a factor of 4.7. The ratio of the quantity of acetic acid produced to the amount of glucose metabolized is proportional to the amount of ATP produced (Hempfling and Mainzer, 1975). Using an average value of 72 mmol acetic acid produced per 100 mmol glucose metabolized (Table 5, Run A) a value of 272 mmol ATP per 100 mmol glucose can be calculated.

E. Mass Transfer Analysis of Growth in Phase 2 and Phase 3.

The pattern of mass accumulation in phases 1, 2 and 3 in three experiments conducted at different glucose provision rates is shown in Figure 13. The pattern of mass accumulation in phase 2 and phase 3 indicated that the growth rate in phase 2 ($r_2$) and phase 3 ($r_3$), as well as the mass at the end of phase 2 ($X_{2,3}$), increased as the GFR was increased. These three experiments are shown to display the mass accumulation
Table 7. Glucose Fermentation Products Produced by *Escherichia coli* B Grown in the Maintenance Energy Fermenter.

<table>
<thead>
<tr>
<th>Run</th>
<th>Phase</th>
<th>Hr</th>
<th>Products</th>
<th>a.b.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ethanol</td>
<td>Acetic Acid</td>
</tr>
<tr>
<td>Ad</td>
<td>2</td>
<td>12.25</td>
<td>72</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td></td>
<td>65</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td></td>
<td>86</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>36</td>
<td>93</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47</td>
<td>86</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>103</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>71.5</td>
<td>98</td>
<td>70</td>
</tr>
<tr>
<td>Be</td>
<td>2</td>
<td>29</td>
<td>78</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>42</td>
<td>75</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>68.5</td>
<td></td>
<td>76</td>
<td>74</td>
</tr>
</tbody>
</table>

a. Expressed in millimoles per 100 mmol of glucose fermented.
b. Lactic acid concentration below detection limit \( \leq 0.1 \) \( \mu \)mol (ml)\(^{-1}\) of procedure. Formic acid was not determined.
c. Product ratios for ethanol:acetic acid:succinic acid in phase 1 (Run A) were: 1:0.82:0.12 at 7.25 h and in phase 1 (Run B) were: 1:1.04:0.11 at 6.0 h. For comparison, the ratio in phase 2 (Run A) was 1:0.85:0.20 at 22 h and in phase 3 (Run A) was 1:0.71:0.24 at 71.5 h. Comparable figures for Run B were 1:0.84:0.29 at 29 h and 1:0.97:0.33 at 68.5 h.
d. GPR of 162 \( \mu \)mol glucose (h)\(^{-1}\), \( F_R \) was 23 ml(h)\(^{-1}\).
e. GPR of 752 \( \mu \)mol glucose (h)\(^{-1}\), \( F_R \) was 105 ml(h)\(^{-1}\).
Fig. 13. Relationship between bacterial mass accumulation and GPR when three different values of the glucose-1 provision rate of 1503 μmol(h)^{-1} (o), 782 μmol(h)^{-1} (□), and 476 μmol(h)^{-1} (△) were used. The value of S_p in each experiment was 6.94 μmol(ml)^{-1}. The closed symbols represent the mean of three replicate samples. The length of the bar represents the range of the three values.
Bacterial dry wt (mg(h)^{-1})

Post inoculation time (h)

Fig. 13
over a wide range of glucose provision rates. The general pattern of mass accumulation has been verified in 19 other experiments. The inflection points between phases 1 and 2 and between phases 2 and 3 were determined by extrapolating the lines in the various phases to the intersection point with the lines of the appropriate phase. The extrapolation was found to be an accurate determination of the mass that occurred at the inflection point. Other experiments (Figure 18) show that the extrapolation is valid.

The rate of mass accumulation in phases 2 and 3 plotted against GPR is shown in Figure 14. The line which related \( r_2 \) to GPR was determined by linear regression. Since the deviations of the data points from the regression line were large, a t-test (Snedecor and Cochran, 1967) was conducted to determine if the line could be fitted through the origin. The t-value was 0.50 which is not significant at the 5% level with 11 degrees of freedom. Therefore, a linear line which relates \( r_2 \) to GPR can be fitted through the origin. This relationship can be described by the equation:

\[
 r_2 = d(S_R F_R)
\]

where \( d \) is the regression coefficient and has a value of 0.0208 mg bacterial dry weight (umol glucose)\(^{-1}\). From the definition of growth yield (biomass produced per amount of substrate metabolized) it follows that \( Y_a \) in phase 2 is equal to \( d \) and is constant in phase 2.

Inspection of the data in Figure 14 indicates that the relationship between \( r_2 \) and GPR is curvilinear in nature.
Fig. 14. Relationship between rate of bacterial mass accumulation in phase 2 (○) and phase 3 (●) and glucose provision rate. Each data point represents the growth rate in the respective phase in different experiments.
Growth rate (total fermenter mass change in mg(h)^{-1})

Glucose provision rate (umol glucose (h)^{-1})
The least complicated curve that can be fitted to the data is a simple exponential function. A linear regression of logarithmically transformed data results in the following equation:

\[ r_3 = a(S_{RF_R})^b \]

where \(a\) is the antilog of the ordinate intercept and has the value of \(7.18 \times 10^{-4}\) mg bacterial dry weight (\(\mu\)mol glucose\(^{-1}\)) and \(b\) is the linear regression coefficient and has the value of 1.41.

The growth rate in general in either phase 2 or phase 3 can be described as:

\[ r = Y_a(S_{RF_R}) \]

where \(Y_a\) is the apparent cell yield and defined as \(\Delta x/\Delta s\) where \(\Delta x\) is the change in mass (mg) per time and \(\Delta s\) is the amount of substrate (\(\mu\)mol) consumed per time. The value for \(Y_a\) in phase 3 can be calculated from the combination of equations 24 and 25. The resulting equation is:

\[ Y_a = a(S_{RF_R})^{b-1} \]

and is graphically displayed in Figure 15. Unlike \(Y_a\) in phase 2, \(Y_a\) in phase 3 follows a curvilinear function of GPR.

The plot of the total fermenter mass \((X_{2,3})\) in mg vs GPR (Figure 16) indicated a linear relationship between these two variables. The value of \(X_{2,3}\), e.i. the mass of the population as it enters the maintenance energy phase, should be determined by the maintenance energy demands of the population. If this is the case, \(X_{2,3}\) should be identical to the
Fig. 15. The dependence of the apparent growth yield in phase 3 on glucose provision rate. The curve was calculated from equation 26.
FIG. 15

$Y_a$ (mg bacterial dry wt (µmol glucose)$^{-1}$)

Glucose provision rate (µmol(h)$^{-1}$)
Fig. 16. Relationship between total cell mass in the fermenter at the end of phase 2 and glucose provision rate. Each data point represents a single determination of $X_{2,3}$.
maximum mass obtainable in a system where the populations' maintenance energy requirements ultimately limit the population size. The maximum population mass can be related to the maintenance demands of a population by the relationship given in equation 18. This equation is valid under conditions in which the residual substrate concentration in the fermentation vessel is zero. If \( X_{2,3} \) is taken as equivalent to \( X_{\text{max}} \) and \( q_a \) as the assimilation coefficient actually observed in phase 2 (see following section), the \( X_{2,3} \) can be used to calculate the maintenance energy coefficient of a population at the end of phase 2. Using the slope of Figure 16 (0.577 mg bacterial dry wt (μmole glucose)\(^{-1}\)) and a \( q_a \) ranging from 0.05 to 0.135, an \( m_c \) ranging from 0.00165 to 0.00145 μmol glucose (μg bacterial dry wt)\(^{-1}\)(h)\(^{-1}\) can be calculated. These values for the \( m_c \) are approximately 50 to 70% greater than the value determined (0.00096 μmol glucose)(μg bacterial dry wt)\(^{-1}\)(h)\(^{-1}\) in the chemostat.

F. Assimilation of Substrate Carbon into Cell-bound Carbon.

The amount of substrate carbon that is incorporated into cell carbon can be calculated on the basis of cell yield if the fraction of substrate and the fraction of biomass that is carbon is known (Stouthamer and Bettenhausen, 1973). Since the glucose concentration in the fermenter is essentially zero in phases 2 and 3 and \( r_2 \) and \( r_3 \) are constant in their respective phases, hence the rate of assimilation is constant, the rate of change of bacterial mass in both phase 2 and phase 3 can be used to calculate the assimilation coefficient (\( q_a \)).
amount of substrate carbon assimilated is determined from the relationship:

\[ \Delta S = \Delta S_c + \Delta S_E \]

where \( S \) is the amount of substrate utilized, \( \Delta S_c \) is the amount of substrate utilized to provide cell carbon, and \( \Delta S_E \) is the amount of substrate utilized to provide energy. The parameter \( \alpha_a \) is related to \( \Delta S_c \) by the ratio:

\[ \frac{\Delta S_c}{\Delta S} = \alpha_a \]

If equation 27 is divided by \( \Delta X \) (change in total fermenter mass per time) and the following substitution made: \( \Delta X/\Delta S = Y_a, \Delta X/\Delta S_c = Y_c, \) and \( \Delta X/\Delta S_E = Y_E, \) equation 27 can be expressed as:

\[ \frac{1}{Y_a} = \frac{1}{Y_c} + \frac{1}{Y_E} \]

where \( Y_a \) is the apparent growth yield, \( Y_c \) is the growth yield in terms of carbon assimilation and \( Y_E \) is the growth yield in terms of energy utilization. \( Y_c \) and hence \( \Delta S_c \) can be determined by use of the relationship:

\[ Y_c = \frac{\Phi}{Y} \]

where \( \Phi \) is the fraction of substrate that is carbon and \( Y \) is the fraction of biomass that is carbon. The value of \( Y \) for \textit{E. coli} B was determined as 0.456 \( \mu gC(\mu g \text{ cell dry weight})^{-1} \) in cells utilizing a variety of carbon sources over a large range of specific growth rates (Hempfling and Mainzer, 1975).
The above equations were used to calculate the percent assimilation ($q_a$ expressed as a percent) of substrate carbon in phase 2 and phase 3 over a range of glucose provision rates. These results are shown graphically in Figure 17. Assimilation in phase 3 and phase 2 followed a curvilinear function of glucose provision rate where assimilation increased as glucose provision rate increased. Assimilation in phase 2 reached a limit value of approximately 13% at substrate provision rates above 400 $\mu$mol glucose(h)$^{-1}$. This maximal limit of assimilation of substrate carbon approximated the percentage of incorporation (13.5%) calculated for exponential growth in the chemostat. The value of $q_a$ for exponential growth was calculated as above using the data presented in Table 3. An average value for $q_a$ of 13.2% in phase 2 can also be calculated from the value of $Y_a$ which was obtained from equations 24 and 25. This value for $q_a$ is approximately equal to the values obtained above for the maximal value in phase 2 and for exponential growth. Since $Y_a$ is a constant in phase 2, $q_a$ should also be a constant over a wide range of substrate provision rates in phase 2.

G. Response of a Maintenance Energy Phase Culture to an Increase in GPR.

The response of a maintenance energy phase culture to a fourfold increase in GPR is shown in Figure 18. The GPR was increased to 1500 $\mu$mol(h)$^{-1}$ by increasing $F_R$ from 55.8 ml(h)$^{-1}$ to 216 ml(h)$^{-1}$ at 69.25 h or 45 h into phase 3. The culture responded immediately to the increased supply of
Fig. 17. Dependence of percent assimilation in phase 2 (○) and phase 3 (●) on glucose provision rate. Each data point represents one determination from the rate of mass change in the respective phases. Percent assimilation was calculated from equation 28.
Figure 17

Percent assimilation vs. Glucose provision rate (μmol(h)^{-1}).

- O: Data points
- •: Estimated curve
Fig. 18. Response of bacterial dry weight (○), cell numbers (■), DNA (▲), RNA (□) and protein (▲) in a maintenance energy phase culture to a four-fold increase in GPR. The pre-shift-up $F_R$ was 55.8 ml(h)$^{-1}$ and the shift-up $F_R$ was 216 ml(h)$^{-1}$. 
glucose. The rate of change of bacterial dry weight, protein, RNA and cell numbers increased without a perceptible delay. The amount of DNA was an exception to this pattern. The rate of DNA accumulation remained unchanged during the initial 8 h, after which time the rate of DNA accumulation increased.

Bacterial numbers, dry weight and protein increased between 30 and 40 percent during the first 6 h of increased GFR. The increase in RNA was at least 2 times the increase in bacterial dry weight, protein and bacterial numbers during the initial 6 h of increased GFR.

During the shift-up, glucose was provided at a rate which was in excess of the glucose necessary to meet the maintenance energy requirements of the population that existed at the time of shift-up. Therefore, the rate of macromolecular accumulation during the shift-up would be expected to be proportional to the rate of accumulation that existed in phase 2. The rates of macromolecule accumulation in phase 2 and 3 and in the shift-up are summarized in Table 8. The rate of increase of bacterial dry weight during the initial 6 h of the shift-up was exactly 4 times and protein 3 times the rate of accumulation of the respective macromolecules during phase 2. The rate of RNA accumulation during the shift-up (initial 6 h) was 8 times the phase 2 rate.

Dry weight, protein and bacterial numbers all followed a hyperbolic pattern of increase during the shift-up as opposed to the linear rate of increase seen in phase 2.
Table 8. Change in Culture Parameters When a Phase 3 Culture was Exposed to a
Four-fold Increase in GPR\(^a\).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Percent Increase in Culture Parameter(^b)</th>
<th>Rate of Change of Parameter(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>During the Shift-up</td>
<td>Phase 2</td>
</tr>
<tr>
<td>Dry Weight</td>
<td>43</td>
<td>14.5</td>
</tr>
<tr>
<td>Protein</td>
<td>38</td>
<td>13.5</td>
</tr>
<tr>
<td>RNA</td>
<td>87</td>
<td>1.6</td>
</tr>
<tr>
<td>DNA</td>
<td>5</td>
<td>1.2</td>
</tr>
<tr>
<td>Bacterial Numbers</td>
<td>30</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Phase 3 GPR was 387 \(\mu\text{mole(h)}^{-1}\) at a \(F_R\) of 55.8 \(\text{ml(h)}^{-1}\). At 45 h into phase 3, \(F_R\) was increased to 216 \(\text{ml(h)}^{-1}\).

\(^b\)Applies to first 6 h of increased GPR.

\(^c\)Expressed as \(\mu\text{g(ml)}^{-1}\)(h)\(^{-1}\).

The loss of cellular macromolecules was followed by radiolabelling cells with either $^3$H-thymidine, $^{14}$C-glucose or $^{14}$C-leucine. The loss of cell-bound carbon was determined by adding $^{14}$C-glucose to the fermentation vessel during the appropriate phase and following the loss of radioactivity from the cells. The rate of loss of $^{14}$C-carbon from the cell was calculated from the relationship:

$$d_{pl}(ml)^{-1}_{T_1} = d_{pl}(ml)^{-1}_{T_0} e^{-dt}$$

where $d_{pl}(ml)^{-1}_{T_1}$ is the concentration of cell-bound radioactivity at time $1$, $d_{pl}(ml)^{-1}_{T_0}$ is the concentration of cell-bound radioactivity at time $T_0$, $d$ is the rate of loss of radioactivity per hour, $t$ is the time interval, in hours, between $T_1$ and $T_0$ and $e$ is the base of the natural logarithms. The value of $d$ was expressed as a percentage to obtain the percent loss of radioactively labeled compound per h. This formula was used to calculate all succeeding rates of radiolabel loss. The data expressed in Figure 19 indicated that the rate of loss of $^{14}$C-labeled carbon was a function of growth phase. The rate of carbon lost per h increased as the cells entered phase 3. During phase 3, the rate of carbon lost per h showed a curvilinear pattern with the rate of loss decreasing late in phase 3. The rates of loss of cell-bound carbon per h are summarized in Table 9 for a series of experiments conducted at a range of glucose provision rates.
Fig. 19. Loss of $^{14}$C-labeled carbon from cells in phase 2 and phase 3. $^{14}$C-glucose was added at a time corresponding to the arrow. GPR was 387 μmol(h)$^{-1}$ at an Fr of 56 ml(h)$^{-1}$. The bars represent the range in values obtained from two determinations. The location of the phases was determined on the basis of bacterial dry weight data.
Table 9. Loss of Cell-Bound Carbon from Maintenance Energy Fermenter Cultures of *Escherichia coli* B.

<table>
<thead>
<tr>
<th>Glucose Provision Rate (µmol(h)^-1)</th>
<th>Percent Loss of Cell-Bound Carbon (h)^-1</th>
<th>Phase 2</th>
<th>Phase 3 Early[^a]</th>
<th>Phase 3 Late[^b]</th>
<th>Shift-up[^c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1500</td>
<td></td>
<td>-</td>
<td>-</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>1250</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>400</td>
<td>0.4</td>
<td>-</td>
<td>1.2</td>
<td>3.5</td>
<td>-</td>
</tr>
<tr>
<td>430</td>
<td>0.6</td>
<td>1.2</td>
<td>0.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>390</td>
<td>0.4</td>
<td>1.3</td>
<td>0.8</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

[^a]: Early phase 3 rates were calculated in the initial 15 h of phase 3.
[^b]: Late phase 3 rates were calculated after 50 h.
[^c]: Substrate provision rate was increased late in phase 3 to approximately 4 times the value used throughout phases 1, 2 and 3.
The change in rate of cell-bound carbon loss between phase 2 and phase 3 was found to be reproducible as was the curvilinear pattern of loss in phase 3. The rate of loss of carbon in phase 3 did not appear to be a function of substrate provision rate. In a shift-up experiment, the rate of carbon loss late in phase 3 was found to increase from $1.2\% (h)^{-1}$ to $3.5\% (h)^{-1}$ when the glucose provision rate was increased by a factor of 4 times that of the value used throughout phases 1, 2 and 3. Therefore, this rate is proportional to the increase in the rate of glucose provision (4 times).

The rate of loss of DNA and protein was determined by following the disappearance of $^3$H-thymidine and $^{14}$C-leucine from maintenance energy fermenter cultures which had been labeled with these compounds during phase 1. These results are shown graphically in Figure 20 and a summary of the rates of loss occurring in the different phases is shown in Table 10. The rate of loss of DNA as measured by the disappearance of $^3$H-thymidine remained constant throughout phase 2 and phase 3 at a rate of $1.9\% (h)^{-1}$. The rate of protein loss also remained constant in phase 3 at a rate of $1.1\% (h)^{-1}$.

The rate of appearance of diphenylamine reactive material in the filtrate (Figure 11a) can be used to approximate the rate of loss of cellular DNA assuming diphenylamine reactive material in the filtrate represents loss of DNA from the cell. If this assumption is valid, the rate of loss of DNA as measured by the loss of $^3$H-thymidine should approximate
Fig. 20. Loss of cellular DNA (\(^3\)H-label) (upper line) and protein (\(^{14}\)C-label) (lower line) from a maintenance energy fermenter culture of *Escherichia coli* B. Radiolabels were added at the arrow. Experimental conditions were identical to Fig. 18. Each bar represents the range of values obtained from two determinations. The location of the phase changes were determined on the basis of bacterial dry weight data.
Phase I  Phase 2  Phase 3  Shift-up

Post inoculation time (h)

Cell bound radioactivity (log$_0$ dpm ml$^{-1}$)
Table 10. Loss of DNA and Protein From Maintenance Energy
Fermenter Grown Cells of *Escherichia coli* B.

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Percent Loss of Macromolecules per hour</th>
<th>DNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 2</td>
<td></td>
<td>1.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Phase 3</td>
<td></td>
<td>1.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Shift-up</td>
<td></td>
<td>5.6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

*Glucose provision rate was increased late in phase 3 to approximately 4 times the value used throughout phases 1, 2 and 3.*
the rate of loss of DNA as measured by diphenylamine reactive material. At a GPR of 390 μmol(h)^{-1}, the rate at which the experiment in Figure 20 was conducted the rate of DNA secretion or loss of diphenylamine reactive material in phase 3 (Figure 12) would be 1 mg(h)^{-1}. Using a value of 1.9% (h)^{-1} calculated from the data in Figure 20 and a total amount of DNA in the fermenter of 11.3 mg at 60 hours post-inoculation time, a rate of 3H-labeled DNA loss of 0.2 mg(h)^{-1} is obtained. Since the value of the secretion rate obtained from the tritium-labeled DNA is one-fifth the value obtained from the filtrate DNA, DNA precursors are synthesized and secreted before being incorporated into DNA.

The pattern of cellular carbon loss (Figure 19) was different than the pattern of cellular DNA and protein loss (Figure 20). Both DNA and protein were lost as a simple exponential function where cell carbon was lost in a more complicated manner with the rate of loss increasing as the cells enter phase 3 and then decreasing as the cells progress through phase 3.

Shift-up experiments indicated a substantial increase in the rate of loss of cellular DNA and protein when the glucose provision rate was increased by a factor of 4 times in late phase 3. DNA loss increased by a factor of 3 times from 1.9% (h)^{-1} in late phase 3 to 5.6% (h)^{-1}, and subsequently decreased to 3.2% (h)^{-1} during the period of increased glucose provision rate. The loss of cellular protein showed a constant rate during the shift-up and increased by a factor of 2.7 times from 0.9% (h)^{-1} in late
phase 3 to 2.4% (h)^{-1}. The increase in the rate of loss of protein (2.7 times) and DNA (3 times) was comparable to the increase in the rate of loss of carbon (3 times) in experiments under similar conditions.

I. Induction of the Lactose Operon in Maintenance Energy
Phase Cultures of Escherichia coli B.

The inducibility of the lactose operon was used to investigate operon control and protein synthesis in E. coli B cultures growing in the maintenance energy fermenter. The time course of continuous lactose operon induction in a phase 3 culture of E. coli B is shown in Figure 21. The gratuitous inducer, isopropyl-β-thiogalactoside (IPTG) was added 22 hours after the start of phase 3 in an amount sufficient to bring both the medium reservoir and the fermentation vessel to 10^{-3} M in IPTG. The inducer at this concentration is saturating, i.e., the rate of β-galactosidase production is independent of inducer concentration (Novick and Weiner, 1957). As can be seen in Figure 21, the rate of β-galactosidase production 260 units(h)^{-1} increased to a maximum value upon inducer addition and remained at that value throughout the course of the experiment. Immediately after addition of inducer, the bacterial mass accumulation rate decreased by 58% from 43. μg/ml)^{-1}(h)^{-1} to 1.8 μg/ml)^{-1}(h)^{-1} (Figure 22). Similarly, the rate of protein accumulation decreased 58% to 1.7 μg/ml)^{-1}(h)^{-1} from 4 μg/ml)^{-1}(h)^{-1}. The rates of DNA and RNA accumulation also fell by comparable amounts. The microcolony assay for viability indicated that no cell death occurred
Fig. 21. Production of β-galactosidase in a phase 3 culture of *Escherichia coli* B. A culture 22 h into phase 3 was continuously exposed to $1 \times 10^{-3}$ M IPTG. GPR was 193 μmol(h)$^{-1}$ at an $F_R$ of 57 ml(h)$^{-1}$. Each data point represents the average of duplicate determinations.
B-galactosidase activity (units ml⁻¹ x 10⁻³)

Hours after induction
Fig. 22. Relationship between β-galactosidase synthesis (●) and accumulation of bacterial dry weight (○) and protein (▲) in maintenance energy phase culture of Escherichia coli B. Induction conditions were identical to Fig. 21. IPTG was added at the arrow. β-galactosidase assays were conducted in duplicate and each data point represents an average value.
Figure 22:

Phase 2

Phase 3

Bacterial dry wt and Protein [µg (m)]

β-galactosidase activity (units (m) x 10^-3)

Post inoculation time (h)
during the induction. The specific activity of β-galactosidase was being synthesized faster than other cellular proteins. The rate of increase in β-galactosidase specific activity was not completely linear (Figure 23) and slowly decreased with time indicating that the rate of β-galactosidase synthesis, relative to other cellular proteins, was decreasing.

The actual mass of β-galactosidase synthesized in phase 3 can be calculated from the number relating enzyme units to enzyme weight which, for β-galactosidase, is 400,000 units(mg)−1 (Kennel and Riezman, 1977). During phase 3, β-galactosidase was synthesized at a rate of 260 units per hour which corresponds to a mass rate of 0.65 μg(ml)−1(h)−1 or 38% of all new protein synthesis is attributable to β-galactosidase. This number is even higher when the other proteins of the lactose operon are considered. The mass of all lactose operon proteins can be approximated from the ratio of β-galactosidase:permease:acetyl transferase (4:2:1). Approximately 67% of all cellular protein produced during induction were lactose operon proteins. At the end of 45.5 hours of induction, β-galactosidase was 7.5% and 6.6% of total protein and bacterial dry weight, respectively. Total lactose operon proteins were estimated to account for 11.5% of the total bacterial dry weight.

The response of the lactose operon to the presence or absence of inducer was studied in a maintenance energy culture of E. coli B. At 15 hours into phase 3, IPTG was added to bring the final concentration of inducer in the
Fig. 23. Specific activity of β-galactosidase during the induction of a maintenance energy phase culture of *Escherichia coli* B. Induction conditions were identical to Fig. 21.
Figure 23

β-galactosidase specific activity (units/μg Protein) vs. Hours after induction.
fermentation vessel to $10^{-3}$ M. The addition of inducer at this concentration to the fermentation vessel would allow induction of the lactose operon yet would also allow the inducer to be diluted by incoming growth medium to a non-inducible concentration. The culture was induced a second time, 46 hours after the first induction. The net synthesis of $\beta$-galactosidase during the first induction (Figure 24) displayed a bimodal pattern with the first rate of synthesis being 280 units/(ml)$^{-1}$(h)$^{-1}$ and decreasing to 112 units/(ml)$^{-1}$(h)$^{-1}$ approximately 7 hours after induction. Dilution of IPTG by incoming growth medium during the first 7 hours of induction would have reduced the concentration by 40% and would have reduced the rate of synthesis of $\beta$-galactosidase after 7 hours. Carbohydrate analysis of the filtrate indicated that by 30 hours into the induction the concentration of IPTG was reduced by dilution to nearly zero. At this point the synthesis of $\beta$-galactosidase stopped, indicating a response of the lactose operon to the absence of inducer. The second addition of IPTG, 46 hours after the first, resulted in re-induction of the lactose operon. The rate of $\beta$-galactosidase synthesis $230 \mu g/(ml)^{-1}(h)^{-1}$ during this induction was 18% less than the previous rate occurring in the first 7 hours of the first induction. A bimodal pattern $\beta$-galactosidase synthesis rate did not occur in the second induction and synthesis stopped 21 hours after the induction. Synthesis stopped 32 hours after the first induction. Also, the total amount of enzyme produced during the second induction was less than that produced in the previous one.
Fig. 24. β-galactosidase synthesis in a phase 3 culture which was induced, allowed to become repressed, and then reinduced. IPTG was added at the arrow so as to make the fermenter $1 \times 10^{-5}$ M in IPTG. GPR was 193 $\mu$mol(h)$^{-1}$ at an $F_R$ of 57 ml(h)$^{-1}$. Each data point represents the average of two determinations.
The loss of β-galactosidase in phase 2 and phase 3 cultures of *E. coli* B was determined for use as an index of the loss of a single type of protein. Loss of β-galactosidase in phases 2 and 3 was determined by inducing a culture in phase 2 with lactose, a metabolizable inducer, at a concentration of $10^{-3}$ M. The loss of β-galactosidase from an induced culture of *E. coli* B is shown in Figure 25. Figure 26 indicated that the loss of β-galactosidase from cells of *E. coli* B followed first order exponential kinetics. The loss of β-galactosidase per hour was calculated from the relationship described in equation 15 and was expressed as a percent. The rate of loss of β-galactosidase decreased from a constant rate of 3.5%(h)$^{-1}$ in phase 2 to a constant rate of 0.5%(h)$^{-1}$ in phase 3. The rate of loss of β-galactosidase is approximately 3 times greater in phase 2 than the loss of other cellular proteins. In phase 3, the rate of β-galactosidase loss was one-half that of total protein loss. β-galactosidase loss increased dramatically during a 4.2-fold increase in glucose provision rate in phase 3, to a rate of 11.28%(h)$^{-1}$ or an increase of 24 times. This initial rate of loss decreased to 2.4%(h)$^{-1}$ two h into the shift-up and remained constant for an additional 18 hours. The rate of β-galactosidase loss during the shift-up, when compared to protein, was higher in the first two h of operation but was nearly equal to the protein rate late in the shift-up.
Fig. 25. Loss of β-galactosidase from a phase 2 and phase 3 culture of *Escherichia coli* B. Lactose was added in phase 2 so as to make the fermenter 1 X 10⁻³ M in lactose. The GFR was 256 µmol(h⁻¹) at an FR of 57 ml(h⁻¹). During the shift-up the FR was increased to 237 ml(h⁻¹). Each data point represents the average of two assays. The location of the phases was based on bacterial dry weight data.
Fig. 26. Exponential loss of β-galactosidase from phase 2 and phase 3 cells of Escherichia coli B. Experimental and induction conditions were identical to Fig. 25.
J. Response of a Maintenance Energy Phase Culture to Bacteriophage.

The susceptibility of maintenance energy phase cells of *E. coli* B to virus infection was determined by adding the bacteriophage T\(_4\) to the culture in phase 3. The bacteriophage were added 22.5 h into phase 3 in sufficient numbers to bring the multiplicity-of-infection to 7 bacteriophage per 100 bacteria. The progress of infection was followed by assaying for cell numbers and for viral numbers in the pellet and supernatant fluid (filtrate) fractions of the infected culture. Immediately after infection, cell numbers decreased by 21% and continued to fall at a moderate rate for 12 hours (Figure 27). Culture turbidity paralleled the decrease in cell numbers and fell by 10% in the first 3.5 hours of infection. Thereafter, both turbidity and dry weight increased. An increase in culture turbidity and dry weight with a concomitant decrease in culture viability would indicate an accumulation of dead cells. During the period of rapid cell number and turbidity decrease (the first 3.5 hours of infection) cell associated (pellet fraction) and free (supernatant fluid fraction) virus remained essentially constant or decreased slightly (Figure 28). This pattern of cell death without appreciable viral replication would indicate that virus was effecting cell death by a means other than the traditional lytic cycle.

Chloroform and blender insensitivity of the cell-associated virus (Table 11) indicated that virus was not
Fig. 27. Response of bacterial dry weight (●), cell numbers (■), and culture turbidity (▲) in phase 3 culture of Escherichia coli B to infection by bacteriophage T4. Virus was added at the arrow. The GPR was 425 μmol(h)^{-1} at an F_R of 62 ml(h)^{-1}. 
Fig. 28. Change in cell associated virus (●), extracellular virus (○), and cell numbers (■) during the infection of phase 3 culture by T₂. Culture conditions were identical to Fig. 27. Virus was added at the arrow.
Bacterial numbers ($\log_{10} CFU (mL)^{-1}$)

Viral numbers ($PFU (mL)^{-1} \times 10^{-7}$)

FIG. 28

<table>
<thead>
<tr>
<th>Post-inoculation Time(^a) (hours)</th>
<th>Ratio of PFU(^b)(ml(^{-1})) of Different Fractions(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C014 treated pellet/pellet Blended pellet/pellet</td>
</tr>
<tr>
<td>48.5</td>
<td>1.0</td>
</tr>
<tr>
<td>49.5</td>
<td>0.86</td>
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<tr>
<td>52</td>
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<td>54</td>
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<tr>
<td>72</td>
<td>0.41</td>
</tr>
<tr>
<td>79</td>
<td>0.19</td>
</tr>
<tr>
<td>96</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\)Virus was added at 48 h.

\(^b\)PFU - plaque forming units.

\(^c\)Viral samples taken from maintenance energy fermenter were centrifuged and viral numbers determined on the pellet fraction after one of the following treatments: none, chloroform addition or blending.
irreversibly adsorbed to the cell and that if virus DNA was injected into the cell, it was not dormant and was capable of replicating.

If virus was not replicating in the first 12 hours of infection, dilution would have reduced the average initial concentration of free virus from approximately $3 \times 10^7$ plaque-forming-units (PFU) per ml to $6.4 \times 10^6$ PFU (ml)$^{-1}$. Since this did not occur and free virus remained at approximately $2 \times 10^7$ PFU (ml)$^{-1}$, $1.2 \times 10^{10}$ virus must have been produced as a result of viral replication. If during the initial 12 h of infection, each cell death produced virus, the average yield of virus per infected cell would be 60. If the original 21% decrease in cell numbers during the first 3.5 h of infection did not result in virus production, the average yield of virus produced per infected cell would be approximately 170.

The period of reduced viral replication, where virus was replicating at a rate just sufficient to counter the effects of dilution, continued for approximately 22 hours after infection. At this point, most viral particles began replicating, as indicated by increased chloroform sensitivity (Table 11), and by 26 hours after infection the virus could effect massive lysis of the culture. During the time period 70 to 79 h, the period of most rapid cell lysis, a total of $6.3 \times 10^{10}$ virus was produced. This value was calculated by adding the number of cell-associated virus produced during this time period to the number of free (filtrate) virus which had been corrected for washout. Since $7.4 \times 10^8$ cells died
during this time period, the yield of the number of virus produced per cell death would be $85$. By 36 hours after infection, virus had lysed $99.9\%$ of the culture. Since virus sensitive cells were lysed by 36 hours after infection, cells resistant to virus infection could have replicated and brought about the increase in bacterial numbers and turbidity which occurred after the period of general lysis.

K. Effect of Penicillin on Maintenance Energy Phase Cells of Escherichia coli B.

One of the consequences of the relationship stated in equation 16 is that once the maintenance energy demands of a population become equal to the amount of glucose available, the population ceases growth and becomes dormant. To ascertain whether cells in phase 3 were dormant, penicillin was added to phase 3 cultures of E. coli B. Penicillin was added 22 hours into phase 3 in an amount such that the penicillin concentration in both the medium reservoir and fermentation vessel was 100 units(ml)$^{-1}$. Approximately 30 minutes after penicillin addition cell numbers and culture turbidity decreased exponentially (Figure 29). By 8 hours after addition, cell numbers had decreased by $99.97\%$. During this same period, culture turbidity decreased by $44\%$. Cell numbers and turbidity continued to decrease for as long as these parameters were monitored.
Fig. 29. Response of cell numbers (■) and culture turbidity (▲) to the addition of penicillin. Penicillin was added during phase 3 at 47 h in an amount sufficient to bring both the fermenter and medium reservoir to 200 units (ml)⁻¹. GPR was 429 μmol(h)⁻¹ at an F_r of 62 ml(h)⁻¹.
A continuous culture apparatus with total biomass feedback was assembled to investigate the biosynthesis and regulation in a maintenance energy phase culture of \textit{E. coli}. Theoretical analysis (equations 16 and 17) of this system indicated that ultimate population mass in the culture when grown under energy-limiting (glucose) conditions would be determined by the populations' maintenance energy requirements. Schultz and Gerhardt (1969) stated that such a maintenance-energy population would be extremely useful in revealing information about maintenance energy metabolism.

Cell growth in the maintenance energy fermenter was found to follow three sequential phases. The existence of these three phases was predicted by mass transfer analysis. The limit of growth in phase 1 coincided with an $P_S$ value of $5.6 \times 10^{-3} \mu\text{mol(ml)}^{-1}$ which is comparable to the reported concentration $5 \times 10^{-3} \mu\text{mol(ml)}^{-1}$ of glucose at which the growth of \textit{E. coli} becomes glucose dependent (Shehata and Marr, 1971).

The observed linear glucose-dependent growth in phase 2 was in direct contrast to the predicted hyperbolic nature of growth as indicated by equation 17. The accuracy of
equation 17 in describing \( \frac{dx}{dt} \) in phase 2 can be tested by sequentially approximating \( \frac{dx}{dt} \) after phase 1 and summing these mass changes over the duration of phase 2. This sequential approximation was applied to the data of one of the experiments \( \overline{CPR} = 1503 \ \text{umol}(ml)^{-1} \) presented in Figure 13. The values used for \( m_c \) and \( Y_{\text{max}} \) in equation 17 were those determined in the chemostat. The cell concentration at \( X_{2,3} \) predicted by equation 17 was \( 1.6 \ \text{mg}(ml)^{-1} \) compared to an observed value of \( 1.9 \ \text{mg}(ml)^{-1} \). Thus, equation 17 underestimated the value of \( X_{2,3} \) by 19%. The accuracy of this prediction depends in a large way on the accuracy of the chemostat-determined values of \( m_c \) and \( Y_{\text{max}} \) which were 0.00096 \( \mu \text{mol glucose (pg dry weight)}^{-1}(h)^{-1} \) and 28.9 \( \mu \text{g dry weight(\mu mol glucose)}^{-1} \), respectively. For comparison, values of \( m_c \) and \( Y_{\text{max}} \) of 0.0054 \( \mu \text{mol glucose (pg dry weight)}^{-1}(h)^{-1} \) and 42.8 \( \mu \text{g dry weight(\mu mol glucose)}^{-1} \), respectively, can be calculated from the data of Mainzer and Hempfling (1976) for \( E. \coli \) B grown in anaerobic continuous culture under similar cultural conditions. At this time, the disparity between the two sets of values can not be explained. The disparity may be due to differences in the two strains of bacteria or in medium composition. The composition of the growth medium has been reported to have a strong influence on \( m_c \) and \( Y_{\text{max}} \) (Stouthammer and Bettenhausen, 1973).

The underestimation of \( X_{2,3} \) by equation 17 can be attributed to the term \( (\frac{-m_c}{X}) \). This term corrects the amount of glucose available for growth for the amount of glucose necessary to maintain the population at any one time. Since
$\frac{dx}{dt}$ did not decrease as predicted by equation 17, the maintenance requirements in phase 2 may be independent of cell mass. The other possible consequence of a constant $\frac{dx}{dt}$ in phase 2 when considering equation 17 is that $m_c$ could be continuously decreasing, and the amount of glucose available for growth would remain constant, which would result in a constant value of $\frac{dx}{dt}$. Although $\frac{dx}{dt}$ is constant, the specific growth rate ($\mu$) as calculated by equation 5 is continuously decreasing in phase 2. Since $\mu$ is decreasing in phase 2 and since the value of $m_c$ has been reported to vary directly with the specific growth rate (Neijssel and Tempest, 1976a), the assumption that the value of $m_c$ is continuously decreasing in phase 2 may be a valid one.

The rate of growth in phase 2 was found to be directly proportional to the rate at which glucose was provided to the culture (Figure 14 and equation 24). This direct proportionality may be explained in light of the evidence that the transport of glucose is the growth rate limiting step in E. coli and that the activity of the phosphotransferase system is regulated according to the growth rate (Kornberg and Jones-Mortimer, 1977).

Equations 16 and 17 predicted that the period following phase 2, when the amount of energy equaled the amount of energy needed for maintenance, would be a steady state of constant mass without growth. The observed accumulation of mass in phase 3 (Figure 4) indicated that this phase was not a period of constant mass. The observed growth and mass accumulation
in phase 3 could conceivably be the result of: 1) a fraction of the population dying and releasing the amount of energy they used for maintenance to other members of the population for use as growth energy, 2) the maintenance energy requirements of the population becoming independent of cell mass in phase 3, or 3) the value of $m_c$ decreasing throughout phase 3 such that the amount of substrate used for maintenance was constant in phase 3 and was less than the total amount of available energy, and 4) the ATP yield per unit amount of glucose fermented becoming greater in phase 3 than in phase 2.

The last hypothesis is easily refuted since the amount of ATP produced during all three phases was identical (Table 7). The data presented in Table 7 indicated that $\mu$ did not alter the fermentation pattern of E. coli grown anaerobically. Therefore, the amount of ATP derived from the fermentation of glucose was independent of growth rate. These results are in contrast with a previously reported study which indicated that in E. coli B, $\mu$ did influence the fermentation of energy substrate and hence, the ATP yield in the organism (Hempfling and Mainzer, 1975). The ratio of 1:0.82:0.12 for ethanol:acetic acid:succinic acid for exponential growth compares favorably to published ratios (1:0.83:0.14) of fermentation end-products in E. coli grown under similar conditions (Belaich and Belaich, 1976).

Several lines of evidence would argue against the first hypothesis. The microcolony technique for assessing the viability of a culture indicated that culture viability never decreased to below 92% during all three phases. The
amount of cell death that is necessary to free a certain amount of maintenance energy for use as growth energy in phase 3 can be calculated from the value of \( r_3 \) of any one run and the value of \( Y_{\text{max}} \) which was obtained from the chemostat data. In the experiment shown in Figure 4, approximately 30% of the population would have died in the first 29 hours of phase 3 growth if the observed rate of growth was due to cell death. Since a population which contained 30% nonviable cells could easily be detected in the microcolony viability assay, hypothesis number one can be rejected. Cryptic growth can also be eliminated as a means of providing energy for cell growth in phase 3 since assays for an intracellular enzyme, lactic dehydrogenase, indicated that extensive amounts of lysis did not occur.

The second hypothesis which stated that the energy requirements of the population are independent of culture mass in phase 3 may be discounted on the basis of the following observation: the start of phase 3 growth always occurs at a critical ratio of cell mass to GPR. The ratio, which can be calculated from Figure 16 is \( 0.587 \text{ mg dry weight/(pmol glucose)}^{-1}(\text{h})^{-1} \). If the maintenance requirements of a population were independent of cell mass, the phase 3 growth would be expected to be identical to the phase 2 growth rate and the culture would not enter phase 3 when the critical mass to GPR ratio was reached.

The third hypothesis, where the value of the \( m_c \) was postulated to decrease throughout phase 3, is the most attractive one. If \( m_c \) were allowed to decrease with a decrease
in $\mu$ in phase 3, the amount of GPR needed to fulfill the requirement for maintenance energy could remain constant. Therefore, the amount of glucose available for growth would remain constant and, hence, $dx/dt$ would be constant in phase 3. Since $\mu$ is continuously decreasing in phase 3, $m_c$ could conceivably decrease. As previously mentioned, Neijssel and Tempest (1976a) reported that $m_c$ may, under some conditions, vary directly with $\mu$.

Analysis of cellular macromolecules during phases 2 and 3 indicated that these two phases were periods of unbalanced growth. Phase 2 cells became reduced in percent composition of RNA and enriched in protein and DNA whereas, phase 3 cells became reduced in percent composition of RNA and DNA and enriched in protein as the respective phases progressed. The sharp change in the rate of increase in dry weight, DNA, RNA, protein and bacterial numbers at the critical mass to GPR ratio which corresponds in time to $X_{2,3}$ indicated that the population adjusts its metabolism to cope with a reduced ratio of available energy to unit culture mass. This change in metabolism is reflected in the reduced carbon assimilation into biomass in phase 3 as compared to phase 2 and exponential growth. The change in metabolism is also evident in the increase in the rate of carbon loss as cells enter phase 3 and in the decrease in amount of LPS sloughed into the culture filtrate during phase 3.

The unbalanced growth in phases 2 and 3 could be a consequence of the cells regulating the rate of macromolecular synthesis to the amount of energy available for growth. The
cellular content of DNA, RNA and protein has been shown to vary with specific growth rate (Wright, Lochart, 1965; Tempest, Herbert, Phipps, 1967). Since the percent composition of DNA and RNA decreased and protein increased as the growth rate was lowered in continuous culture (Tempest, Herbert, Phipps, 1967), the pattern where a culture in phase 3 became depleted in RNA and DNA and enriched in protein may have been a result of a decreasing specific growth rate ($\mu$) in phase 3.

The process by which a culture decreases its RNA content in phase 3 may be analogous to the response of stringent strains of E. coli to amino acid starvation. Since during phase 3 the amount of available energy per cell is low, the intracellular pool of amino acids would be expected to be low. If the intracellular pool of amino acids is low, a stringent cell will respond by reducing the synthesis of RNA, phospholipids, and the lipid A moiety of LPS (Doerr, Apirion, 1978). Therefore, a phase 3 cell may reduce its synthesis of RNA in response to low amounts of available energy. A stringent response in a phase 3 cell would also explain the reduced amount of extracellular LPS in phase 3.

The reduction in percent composition of DNA in phase 3 may be linked to the reduced synthesis of RNA in phase 3. There is some evidence that in E. coli RNA synthesis is necessary for DNA replication (Klein and Bonhoeffer, 1972). The loss of DNA from the cell as measured by $^3$H-thymidine loss and accumulation of diphenylamine-reactive material indicated that the DNA precursors were secreted before being incorporated into DNA. This indicates that the reduced
synthesis of DNA in phase 3 was a result of a process which
blocked precursor incorporation into DNA and that precursor
synthesis was not closely regulated.

The experiments which dealt with the relationship
between the growth rate in phase 3 and GPR (Figure 14)
indicated that $r_3$ would always have a positive value greater
than zero when the GPR was greater than zero. Therefore, a
cell is incapable of distinguishing between energy used for
maintenance and energy used for growth. This idea is also
supported by the conclusion reached by Sykes (1976). He
stated that there is no biochemical mechanism by which a cell
can use intracellular energy totally for maintenance purposes
and none for growth. Since the relationship between $r_3$ and
GPR is definitely curvilinear at glucose provision rates
below 400 $\mu$mol(h)$^{-1}$, the cellular processes which contribute
to maintenance energy may have a greater affinity for intra-
cellular energy supplies than do growth processes. Possibly,
a cell may withdraw energy used for maintenance from the
intracellular energy pool prior to withdrawals of energy
which would be used for growth, but the cell would be unable
to meet maintenance energy demands totally at the expense of
growth energy.

A comparison of the growth yield in phase 2 (obtained
from equation 23) to the growth yields shown in Figure 15
indicated that the growth yield was lower in phase 3 than in
phase 2.

The difference in growth yield and assimilation between
phase 2 and phase 3 may be a reflection of cells inability to
use all exogenous energy for maintenance purposes. Since $dx/dt$ is less in phase 3 than in phase 2, the fraction of GPR that is used for maintenance purposes must be greater in phase 3 than in phase 2. Therefore, a reduced amount of energy when compared to phase 2 is available for incorporation of glucose carbon into cell bound carbon. As a result, both the assimilation coefficient and growth yield are lower in phase 3 than in phase 2. The dependence of the assimilation coefficient in phase 3 on GPR and hence, the growth yield, is a consequence of the increase in the fraction of GPR used for maintenance at low values of GPR.

The loss of $^{14}$C-carbon cellular label indicated that the rate of loss of carbon increased as the culture entered phase 3. The loss of protein as measured by the disappearance of $^{14}$C-leucine from the cell represents only a net loss of protein since in *E. coli* extracellular amino acids are very rapidly transported into the cell (Nath and Koch, 1970). Therefore, the measured rate of $^{14}$C-leucine loss would represent sloughing of protein from the cell and would not be a measure of protein turnover. The rapid incorporation of free amino acids would also explain the absence of free amino acids in the filtrate. The rate of protein loss in phase 3 may represent degradation of a specific type of protein (stability is energy dependent and degradation is energy independent) which is unstable under the conditions of phase 3 where the amount of glucose available per unit amount of biomass is continuously decreasing in phase 2 and phase 3. The $^{14}$C-leucine shift-up experiment
indicated a species of protein whose degradation was energy dependent. This observation is in agreement with the known mechanisms of protein degradation where aberrant proteins require energy for degradation (Gottesman and Zipser, 1978).

On the basis of the above observations, protein stability and degradation in maintenance energy phase cultures are regulated in response to the availability of intracellular energy supplies.

The loss of DNA as measured by the loss of $^3$H-thymidine appears to be energy dependent in much the same way as protein. This is evidenced by the increase in the rate of DNA loss when GPR was increased by a factor of 4 in phase 3. One notable exception is that the loss of protein as measured by the secretion of protein in the filtrate (Figure 12) appears to be mass dependent whereas DNA does not.

The protein synthesizing capability in maintenance energy phase cultures appears to be under-utilized since protein synthesis and bacterial dry weight increased without a perceptible lag after the GPR was increased 4-fold during phase 3. The under-utilization of the protein synthesizing capability may be one means by which a culture is able to supply both energy used for maintenance and energy used for growth when the total supply of endogenous energy is limited. The under-production of protein would be an energy-conserving process when compared to the synthesis of other cellular macromolecules since the energy expended in protein synthesis is approximately 20 times that of DNA, 4 times that of RNA,
150 times that of lipid and 10 times that of polysaccharide (Stouthamer, 1973).

One noteworthy result of the shift-up was that the increase in bacterial dry weight as well as RNA, protein and bacterial numbers followed a hyperbolic curve as predicted by equation 17. As to why a culture should follow the predicted hyperbolic growth during a shift-up and not during phase 2 is not known at this time.

The response of a maintenance energy phase culture to the presence of IPTG indicated that the culture was capable of producing an inducible enzyme. The inducibility of the lactose operon in a maintenance energy phase culture where the energy supply is glucose would in part be determined by the intracellular adenosine - 3',5'-cyclic monophosphate (c-AMP) concentration. Cyclic AMP has been demonstrated to be a necessary requirement in the synthesis of a number of inducible enzyme (Pastan and Adhya, 1976). The presence of glucose in a culture represses the intracellular level of cyclic AMP and results in catabolite repression (Pastan and Perlman, 1970). Therefore, B-galactosidase would be expected to be subject to catabolite repression whenever glucose was present in the culture medium and inducible only when glucose is exhausted. The exhaustion of glucose in a culture has been correlated with a rise in the intracellular level of cyclic AMP (Buettner et al., 1973).

The inducibility of the lactose operon in phase 2 and phase 3 when exposed to lactose or IPTG can be thought to be the result of increased levels of cyclic AMP when the
intracellular concentration of glucose has decreased to a level insufficient to mediate catabolite repression. The potential for β-galactosidase synthesis in a phase 2 or phase 3 culture which is energy restricted may be a result of one specific type of protein being synthesized at the expense of other proteins. If this is the case, the synthesis of β-galactosidase would not require any additional energy for amino acid synthesis and polymerization other than that already committed to protein synthesis.

The production of β-galactosidase in the phase 3 culture which was continuously exposed to inducer comprised a very large fraction of the newly synthesized protein and after 46 h of induction comprised approximately 7.5% of the total cellular protein. This value is slightly higher than the 6% reported for the β-galactosidase fraction of protein in exponential phase cells (Horiuchi et al., 1962). Since β-galactosidase was a constant fraction of the newly synthesized protein, the synthesis of β-galactosidase was regulated according to the rate of overall protein synthesis.

The growth rate reduction in the phase 3 culture after induction may represent the cost of IPTG transport into the cell. Purdy and Koch (1976) have demonstrated that energy is required for the transport of lactose operon inducers into the cell. The reduction in growth rate has been reported in exponential phase cells which had been induced for β-galactosidase (Novick and Weiner, 1957).

The pattern of induction where a phase 3 culture was induced, allowed to become repressed, and then reinduced
indicated that phase 3 cultures displayed the usual controls which governed the induction and repression of the lactose operon. The reduced rate of β-galactosidase synthesis and reduced plateau of enzyme activity in the second induction, when compared to the first, indicated that phase 3 cells had become altered in their ability to produce an inducible enzyme. Conceivably this represented an "aging" of the culture and a reduction of the capability to synthesize protein.

The observed bimodal pattern of β-galactosidase synthesis in a culture when exposed to a continuously decreasing concentration of inducer may be explained by the observations of Novick and Weiner (1957). They found that the maximum rate of β-galactosidase synthesis occurred at inducer concentrations greater than $10^{-3}$ M. At inducer concentrations below this value, the rate of β-galactosidase synthesis was dependent on inducer concentration. Therefore, the reduced rate of synthesis which occurred approximately 7 h after the addition of inducer was due to a reduction of the inducer concentration to below a saturating level.

β-galactosidase was lost at a rate faster than other cellular proteins in phase 2 and at a rate slower than other proteins in phase 3. Therefore, β-galactosidase may not be a reliable index of total protein loss. β-galactosidase may be a protein whose degradation is energy dependent. During the transition between phases 2 and 3 the rate of β-galactosidase loss decreased which indicated that the reduced level of available energy in phase 3 altered the rate of loss.
During phase 3 α-galactosidase was relatively stable as opposed to the high rate of loss during the shift-up. This evidence indicates that the loss or degradation of α-galactosidase is energy dependent.

The infection and replication of the bacteriophage T₄ in a maintenance energy phase culture was a fairly unique process. After infection, the initial decrease in bacterial numbers and turbidity, without appreciable virus replication, indicated that cell death was mediated by a process other than that which occurs in the traditional lytic cycle. This same type of phenomenon was reported by Doermann (1948). Possibly, the initial decrease in bacterial numbers could be due to lysis of the host cell during the infection process. For this to occur, each infecting virus would have to kill three bacteria to account for the 21% drop in bacterial numbers. This would appear to be unlikely since if lysis was effected by adsorption of the virus and injection of viral DNA, the virus must replicate or a noticeable loss of virus would occur. Although it is possible that if the burst size during this initial infection was greatly reduced, virus replication would not have been noticeable in the plaque-forming unit assay.

The virus yield per cell death (170) during the first 12 hours of infection was higher than previously reported value (30 to 40) for cells grown in a minimal medium (Delbruck, 1940). Calculation of the virus yield per cell death in the later period of the infection is unreliable since virus resistant bacteria could contribute to the population size.
The virus-resistant bacteria, once cell lysis made glucose available, could replicate at a rate which would bring their numbers to a substantial fraction of the overall population size.

The time scale of virus replication and host lysis in the maintenance energy fermenter was greatly expanded compared to the average growth cycle of approximately 20 minutes in exponentially growing cells (Delbruck, 1940). Conceivably, the increased growth cycle could be a function of the doubling time (approximately 15 h) in phase 3. Ellis and Delbruck (1939) have reported that the latent period of virus infection was proportional to growth rate.

The first 21 hours of infection where the number of cell-associated and free virus was essentially constant indicated that virus was incapable of overriding cellular control mechanisms and replicating. Only when sufficient cells had died, thus providing a larger energy supply to the viable cells, could virus replicate and lyse the culture. These results indicate that a maintenance energy phase culture very tightly controls the use and allocation of intracellular energy supplies.

The initial drop in turbidity and bacterial numbers and the subsequent period of approximately constant number of cell associated and free virus during the first 21 hours of infection indicated that the bacterial cell population may be heterogeneous in terms of sensitivity to virus infection. Most likely this represents a physiological heterogeneity in the population. Conceivably the heterogeneity may result
from differences in ability to support virus growth or from differences in receptor sites. The latter explanation is suggested by the attachment site of T4 to *E. coli*. The glucose moiety of the LPS molecule has been shown to be the site of T4 attachment to the outer membrane (Prehm et al., 1976). Since LPS synthesis is postulated to be under stringent control in phase 3 and since glucose is in short supply in phase 3, some members of the population could have a reduced number of attachment sites.

The penicillin sensitivity of phase 3 cells indicated that a large fraction of the cells in phase 3 were actively growing. This conclusion is suggested by the mode of action of penicillin. Penicillin is postulated to inhibit peptido-glycan crosslinking and thus is only active against growing cells (Blumberg and Strominger, 1974). The slightly accelerating logarithmic rate of cell death may be suggestive of a population composed of non-growing and growing cells. Conceivable this pattern of cell death could result from recruitment of cells from a dormant population as a result of energy becoming available for use as growth energy. The death of the growing population would allow glucose to become available to the dormant population. The accelerating rate of death would result from recruitment of a dormant population to the growing population, thus an ever-increasing size would be subject to the action of penicillin.

At the present time the hypothesis that some fraction of the population in phase 3 is dormant is open. If the population is indeed dormant, the contribution of the dormant cells to the population is not known.
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