FEEDBACK CONTROL AND THE CONTINUOUS PHASING OF MICROBIAL CULTURES

by

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To my parents,

for providing the inspiration

ABSTRACT

Continuous phasing is a technique for cell culturing that is able to reveal metabolic responses during the cycle of cell growth and division. This is accomplished by the correspondence between the periodic addition of a limiting nutrient and the time required for the growth of a generation of cells. The utility of the technique was greatly improved by the development of a feedback system to control the period of nutrient addition. The production of the biosurfactant surfactin was studied using Bacillus subtilis ATCC 21332. The feedback control system was effective in optimizing the period of nutrient addition, resulting in an increase in the production rate of surfactin by at least a factor of ten in comparison to batch culture. The inherent stability of the new technique also provided a means for characterizing the dynamic response of microbial cultures to changes in the environment.

RESUME

La culture continue en phase est une technique capable de révéler le cycle de croissance et de division des cellules. Ceci est accompli par la correspondance entre l'addition périodique d'un élément nutritif limitatif, et le temps requis pour la croissance d'une génération de cellules. L'utilité de la méthode a été amélioré grandement par l'addition des éléments nutritifs. La production du biosurfact surfactin par le microorganisme Bacillus subtilis ATCC 21332, a été étudiée. Le système de feedback s'est avéré efficace pour optimiser la période d'addition des éléments nutritifs, résultant en une augmentation de la productivité de surfactin par un facteur de dix en avec la culture discontinue. La stabilité inhérente à la nouvelle technique procure aussi une méthode pour caractériser la réponse dynamique des cultures microbiennes aux changements environnementaux.

TABLE OF CONTENTS

Page

Abstract. i
Résumé. ii
Table of Contents iii
Preface vi
List of Figures viii
List of Tables. xiii

CHAPTER 1 INTRODUCTION 1
- 1.1 Batch Culture 5
- 1.2 Continuous Culture. 11
- 1.3 Cell Synchrony. 15
- 1.4 Continuous Phasing. 18
- 1.5 The Cell Cycle. 25
- 1.6 Coupled Oscillators 30
- 1.7 *Bacillus subtilis* ATCC 21332. 33
- 1.8 Oxygen Transfer 38
- 1.9 Objectives. 40

CHAPTER 2 MATERIALS AND METHODS. 43
- 2.1 Biomass Concentration 43
- 2.2 Viable Cell Count 44
- 2.3 Surfactin Concentration 46
- 2.4 Ammonia Nitrogen Concentration. 49
- 2.5 Dissolved Oxygen Concentration. 51
- 2.6 Thin Layer Chromatography 52
- 2.7 Manganese Concentration 53
- 2.8 Amino Acid Analysis 54
- 2.9 Medium Preparation. 57

Page

CHAPTER 3 APPARATUS. 61
- 3.1 The Cyclone Column Reactor. 61
- 3.2 Ancillary Control Equipment 65

CHAPTER 4 EXPERIMENTATION. 75
- 4.1 Batch Culturing 75
  - 4.1.1 Procedure. 75
  - 4.1.2 Results. 77
  - 4.1.3 Discussion 96
- 4.2 Continuous Phasing with Imposed Cycle Times 102
  - 4.2.1 Procedure. 102
  - 4.2.2 Results. 107
  - 4.2.3 Discussion 135
- 4.3 Continuous Phasing with Feedback Control. . 140
  - 4.3.1 Procedure. 140
  - 4.3.2 Results. 148
    - 4.3.2.1 Minimal starvation. 148
    - 4.3.2.2 Controlled starvation 152
    - 4.3.2.3 Residual nitrogen 165
    - 4.3.2.4 Dissolved oxygen. 168
    - 4.3.2.5 Dynamic response. 173
  - 4.3.3 Discussion 181
- 4.4 Product Identification. 193
  - 4.4.1 Procedure. 193
  - 4.4.2 Results. 193
  - 4.4.3 Discussion 199
- 4.5 Oxygen Transfer Coefficient 200
  - 4.5.1 Procedure. 200
  - 4.5.2 Results. 208
  - 4.5.3 Discussion 223

Page
4.6 Surfactin Production. 225
  4.6.1 Procedure. 225
  4.6.2 Results. 226
  4.6.3 Discussion 229

CHAPTER 5 SYNTHESIS AND CONCLUSIONS. 234

REFERENCES. 241

APPENDICES

A. Method for Quantifying Surfactant Concentration from Measurements of Surface Tension. . . . 257

B. Determination of K_La by the Sulphite Oxidation Method. 260

C. Computer Programme Listings in HP Basic . . 262

D. Chemical Suppliers. 292

PREFACE

This thesis describes the development of a new technique for the culturing of microbial cells. The technique is based on a method called continuous phasing, developed by Dr. P.S.S. Dawson, formerly of the National Research Council of Canada and now retired. The addition of computerized feedback control to the continuous phasing, greatly increased its utility for studying cellular metabolism and accelerated the progression towards process optimization. The novelty and significance of this development has led to the filing of a Patent Application on March 17, 1989, on behalf of the National Research Council of Canada.

Many individuals have contributed towards the successful completion of this project. Dr. Peter Dawson was invaluable; his wealth of knowledge, commitment and patience provided both the inspiration and means for success. Ms. Suzanne Liou provided not only competent technical assistance, but also a genuine interest that helped maintain a creative and harmonious atmosphere within the lab. Dr. David Cooper was always available for discussion, and his dedication to rigorous scientific research was a steadying influence that was much needed, although not always appreciated. Several other members of the department also deserve acknowledgement, including Mr. Jean Dumont, always both friendly and helpful, and the office staff, in particular Ms. Valerie Hubbard and

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The Biotechnology Research Institute of the NRC was instrumental in the success of the project. Gratefully acknowledged is Dr. Maurice Brossard for granting the author leave of absence, Mr. Bernard Gibbs for performing the amino acid analyses, and Dr. Bernard Coupal for vital financial support during difficult times.

Finally, the author greatly benefitted from a collaboration with Dr. Raynalde Laprade of the Université de Montréal. Although not described here, the results of this collaboration provide a good example of the advantages to multi-disciplinary research.

Excellent typing services were provided by Ms. Eileen Penn of Executype.

LIST OF FIGURES

No. Page

1 The Phases of Growth in Batch Culture 7

2 The Effects of Dilution Rate on Continuous Cultivation 14

3 The Principle of Continuous Phasing 21

4 The Structures of Surfactin and Iturin A 36

5 Calibration Curve for Determination of Manganese by Atomic Absorption 56

6 The Cyclone Column Reactor 63

7 Phasing Control by Timer and Level Probes 67

8 Phasing Control by Computer and Electronic Balance 73

9 A Batch Culture of *B. subtilis* in the Original Medium of Cooper et al. (1981) 79

10 A Batch Culture of *B. subtilis* in a Nitrogen-limited Medium Showing the Minimum Dissolved Oxygen 81

11 A Batch Culture of *B. subtilis* in Two Carbon-limited Media 84

No. Page

12 A Batch Culture of B. subtilis Prior to Beginning Continuous Phasing with Nitrogen Limitation 86

13 Variation in Growth During Consecutive Batches with Nitrogen Limitation 90

14 Variation in Nitrogen Utilization During Consecutive Batches of B. subtilis 92

15 Consecutive 20-hour Batch Cultures Using a 1.6% Inoculum 95

16 Consecutive 12-hour Batch Cultures Using a 12.5% Inoculum 98

17 Flow Chart for Control of Continuous Phasing Using Imposed Cycle Times 106

18 Continuous Phasing with Four-hour Imposed Nutrient Cycles with Carbon Limitation 110

19 Six-hour Nutrient Cycles with Carbon Limitation 113

20 Continuous Phasing with Four-hour Imposed Nutrient Cycles with Nitrogen Limitation 117

21 Four-hour Nutrient Cycle Number 41 120

22 Four-hour Nutrient Cycle Number 82 122

No. Page

23 Four-hour Nutrient Cycle Number 100 124

24 Continuous Phasing with 200-minute Imposed Nutrient Cycles 127

25 Cell Washout During 160-minute Imposed Nutrient Cycles 130

26 Continuous Phasing with 180-minute Imposed Nutrient Cycles 132

27 Three-hour Nutrient Cycle Number 78 134

28 Flow Chart for Control of Continuous Phasing Using Feedback 143

29 Continuous Phasing with Feedback Control and Minimal Cell Starvation 151

30 Nutrient Cycles Numbers 14 and 90 from Continuous Phasing with Feedback Control 154

31 Continuous Phasing with Feedback Control of Cell Starvation Period 157

32 Nutrient Cycle Number 19 with a 90 Minute Starvation Period 160

No. Page

33 Nutrient Cycle Number 36 with a 60 Minute Starvation Period 162

34 Nutrient Cycle Number 66 with No Added Starvation Period 164

35 Regular Oscillations in the Dissolved Oxygen Concentration Before and After Addition of a Starvation Period 170

36 Regular Oscillations in the Dissolved Oxygen Concentration Before and After Increasing the Manganese Concentration 172

37 Response of the Cell Population to Step Changes in the Manganese Concentration of Short Duratio . 175

38 Response of the Cell Population to Step Changes in the Manganese Concentration of Long Duration . 177

39 The Phase Plane Portrait of the Dynamic Response to Manganese 180

40 Change in Biomass Concentration During Two Batch Cultures Inoculated from a Phased Culture 188

41 Change in Surface Tension During Two Batch Cultures Inoculated from a Phased Culture 190

42 Apparatus for Determining the Volumetric Oxygen Transfer Coefficient 203

No. Page

43 Change in Dissolved Oxygen Concentration while Gassing Out with Nitrogen 206

44 The Effect of Working Liquid Volume on Oxygen Transfer into Distilled Water 210

45 The Effect of DTAB on Oxygen Transfer 213

46 The Effect of SDS on Oxygen Transfer 215

47 The Effect of Cell-free Broth on Oxygen Transfer. 218

48 The Effect of Broth Concentration on Oxygen Transfer 220

49 The Effect of Broth pH on Oxygen Transfer 222

50 A Method for Determining the Critical Micelle Concentration 259

LIST OF TABLES

No. Page

1 Medium Composition 58

2 Biomass Doubling Times During Batch Growth . . . 88

3 The Effects of the Cell Culture Technique on Specific Growth Rate 114

4 Comparison of Imposed Cycle Times 136

5 Computer Programmes for Control of Nutrient Dosing 146

6 Results from Controlled Starvation Using Feedback Control 155

7 The Effects of Residual Nitrogen on Specific Growth Rate 167

8 TLC of Products from Batch Growth in the Medium of Cooper et al. (1981) 194

9 TLC of Products from Phased Growth in Nitrogen-limited Medium 195

10 TLC of Products from Phased Growth with Various Starvation Periods 197

11 The Results of the Amino Acid Analysis of Various Product Fractions 198

No. Page

12 The Effects of Manganese and Iron on Surfactin Production 227

CHAPTER 1

INTRODUCTION

It is a great advantage for the scientist or engineer in search of new explanations for physical phenomena to be acquainted with certain epistomological principles. This was first realized by physicists and has been elegantly expressed by Eddington (1939) who described the process of observation as "selective subjectivism". Epistomological principles are concerned with the theory of knowledge, defining what is possible to consider as law or fact. One of the most basic epistomological facts is that direct knowledge of physical phenomena is not possible. The method and apparatus used for observation determines what can be observed and provides only an indirect or relative representation of the phenomena under observation. This has very important implications in all scientific inquiries, not just physics. The scientist becomes an active participant in the development of the observations that comprise our model of the physical world. The search for new models is often a result of the limited utility of the existing model from the perspective of practical application, or, correspondence to other models describing seemingly related phenomena. The engineer, although not often responsible for model development (model used in the sense of a scientific paradigm), is perhaps best suited to judge the limitations of a current model's practical application. It is from this perspective that this project was undertaken. It is in attempt to develop an experimental technique that would eventually lead to a new model of microbial growth processes. This new model based on a dynamic perspective of the cell would hopefully be of greater utility to the engineer for the design and control of biological processes.

Engineering of microbial processes differs from most chemical engineering because of the extreme complexity. One liter of fermentation broth typically contains more than a trillion cells, each a fully self-contained entity capable of transforming a few simple chemicals into a replica of itself, often under highly variable conditions. Cellular organization exceeds all chemical processes in complexity by several orders, imparting the ability of self-organization, a feature inherent to life. The size of a bacterial cell is about a micrometer and is therefore very difficult to study in isolation. The amount of material contained within a single cell is extremely small and individual chemical components may often be below the limits of detection. Although far from completed, biochemists have been able to elucidate hundreds of chemical pathways comprising the cellular metabolic processes. However, the complexity of these processes may always defy a complete integrated understanding even for a single organism such as Escherichia coli. Any biological process designed to exploit the capabilities of the cell must, for practical reasons, use many billions of cells simultaneously, thereby achieving a rate of production adequate to satisfy economic constraints. Thus, it is the utilization and control of cell populations that is the basis of the process.

Improvement of a process involves changing a specific parameter and monitoring its effect on performance. If there is only a limited understanding of the mechanisms involved, a purely trial and error approach is required. A methodical experimental programme may eventually reveal a trend, as represented by a more or less continuous relationship between an input and output. This relationship is valid and reflects the reality of the system under observation given the constraints of the experimental methods employed. A very poor degree of resolution, where the underlying phenomena remain hidden, may limit the validity of the relationship to that specific system under those specific conditions. As the degree of resolution increases, so does the perception of the underlying mechanisms and the commonality of phenomena. Concomitant with this is an increase in predictive power within the specific system and also to other systems that manifest the same mechanisms. However, as the degree of resolution increases, so does the perceived complexity and necessary integration to connect the phenomena into a functioning whole. Therefore, the engineer must attempt to reconcile the need for specific information concerning mechanism and the utility of treating the system as a single entity or "black box". Attempting to predict the behaviour of a process when subjected to conditions outside those covered by the specific experiments can only be successful if the underlying phenomena have general application. The relative success of the scientific method for studying physical phenomena demonstrates that many phenomena do have general application if characterized in the appropriate manner.

In this regard, since biochemical engineers must deal with large populations of cells, it is sufficient for purposes of control and prediction if the limit of resolution is confined to cell populations. If the behaviour of the population could be fully characterized, then the level of understanding would be adequate. What is involved in the characterization of an entity such as a population of cells?

The growth and reproduction of the cell is a basic underlying phenomenon in a population of cells. The individual cell is the building block of the population and, therefore, understanding the way cells organize or behave within the population reveals basic truths about the population. In the same way understanding chromosome replication or protein synthesis reveals basic truths about the cell.

By applying the appropriate input signal, it is possible to study phenomena at different levels of resolution. The input signal (or experimental technique) always has some relationship to the phenomena under investigation. It is true that when a population of cells is subjected to a particular environmental condition or stimulus, it will always react in a manner dependent on its underlying mechanisms, regardless of the nature of the stimulus. However, the response will be absolutely dependent on the nature of the stimulus, how the stimulus is perceived and the capabilities of the population to respond. Since the basic building blocks of a population are the individuals, then the stimulus must be perceivable within a range of amplitudes and frequencies that can affect the individual, if understanding the individual is desired.

1.1 Batch Cultures

The early studies concerned with bacterial growth treated a "bacterial culture" as a separate entity, albeit composed of changing numbers of individual cells (Buchanan 1918). The culture medium was a fixed quantity of a nutrient solution into which cells were inoculated. Buchanan (1918) was one of the first to quantify the growth of the cell population and divided the period of the batch culture into seven phases (Figure 1):

i) Initial stationary phase during which the number of bacteria remains constant;

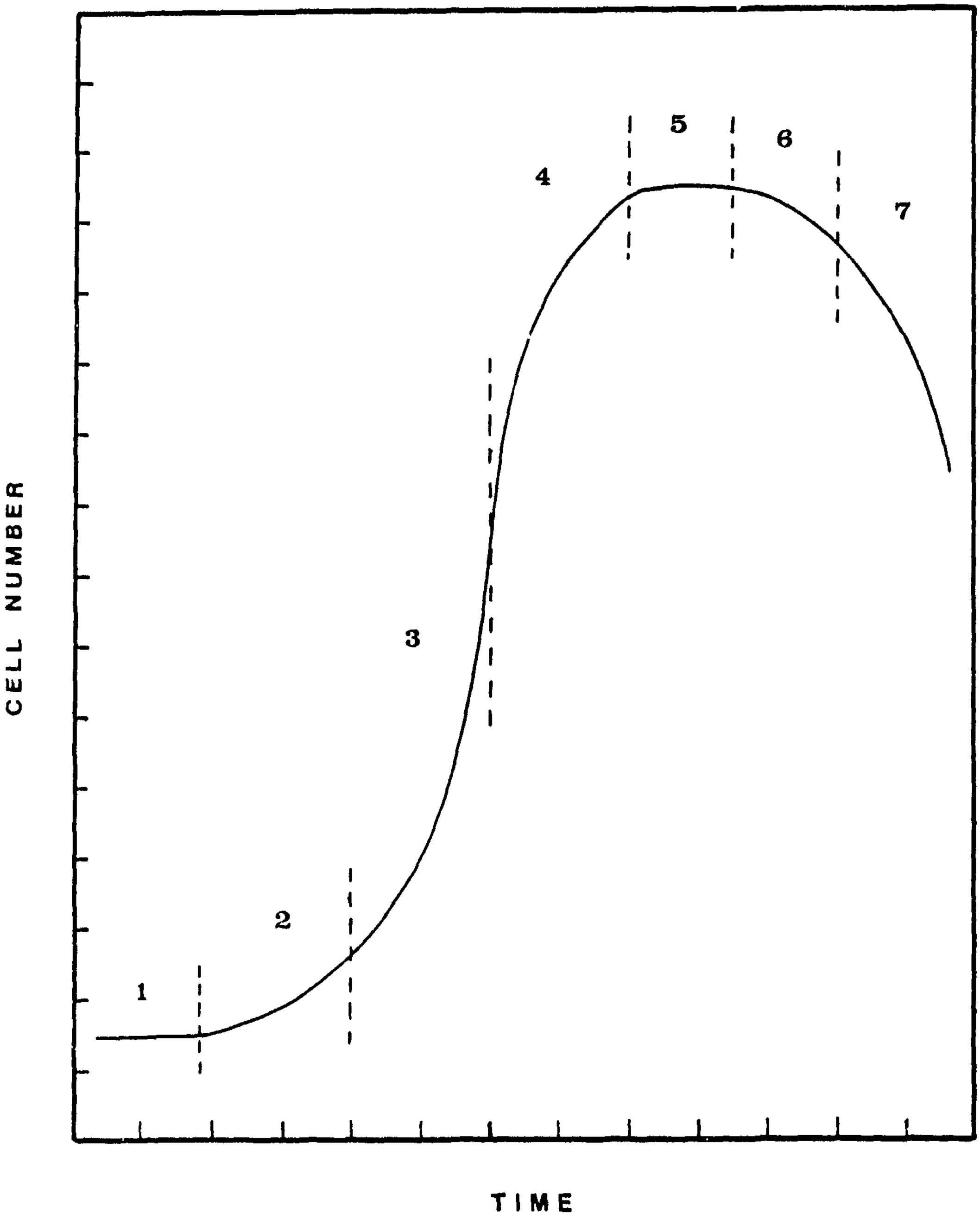
ii) Lag phase during which the cells begin to grow and lasts

Figure 1

The Phases of Growth in Batch Culture

Shown are the seven phases as originally described by Buchanan (1918):

1 - initial stationary
2 - lag
3 - logarithmic growth
4 - negative growth acceleration
5 - maximum stationary
6 - accelerated death
7 - logarithmic death


CELL NUMBER
TIME
1
2
3
4
5
6
7

until all cells are actively growing;

iii) Logarithmic growth phase during which the growth rate remains constant and is maximum;

iv) Negative growth acceleration phase during which the rate of growth decreases until no increase in cell number occurs;

v) Maximum stationary phase during which there is no change in cell number;

vi) Accelerated death phase during which the numbers of bacteria are decreasing at an accelerated rate;

vii) Logarithmic death phase during which the rate of death is constant and maximum.

Buchanan (1918) formulated a general equation to model the change in cell number over the period of the batch culture:

$$b = Be^{\mu kt} \tag{1}$$

where: b is the number of bacteria after time t;

B is the number of bacteria at the beginning of the logarithmic period;

μ is a function of time varying between -1 and 1;

k is the velocity coefficient of growth;

t is time.

It is clear that during the logarithmic growth phase when μ equals one:

$$b = Be^{kt} \tag{2}$$

and K is related to the minimum doubling time (g) by:

$$k = \ln 2/g \tag{3}$$

By varying μ between -1 and 1, all phases of growth and subsequent death can be modelled; however, due to the apparent complexity of the function u with respect to time, it is only when μ equals zero during logarithmic growth and death has the equation found general utility.

The use of batch culture for both the study of microbial growth and commercial product development (particularly ethanol and antibiotics) has persisted to the present with few major new insights. Aiba and Humphrey (1973) in a widely used textbook describe batch culture even more simplistically than Buchanan (1918), reducing the number of phases to four (lag, logarithmic, stationary and death). The major limitation of batch culture is clearly stated: "It is important to appreciate that, in a batch system, the environmental conditions are not constant, even during the phase of constant growth rate...analyses of cells are of little value unless the conditions are precisely defined, but, strictly speaking, it is impossible to do this for a batch culture since conditions change from minute to minute." (Aiba and Humphrey 1973).

In summary, batch culture is a closed system in which the cells grow and divide over many generations (typically 5-10) in an aperiodic but transient nutrient environment. A batch culture usually passes through a number of phases based on growth rate. Interpretation of data from batch culture is problematic for the following reasons:

1) Each generation of cells is subjected to a different environment as the composition of the medium constantly changes.
2) The physiological state or condition of the cells used as the inoculum is not well defined as a result of the source also being a batch culture.
3) A batch culture is a transient phenomenon in which a steady state or equilibrium condition is never obtained.
4) Control of a batch culture is limited to defining the initial composition of the medium and the end point of the fermentation.

These limitations of the batch culture technique result in generally poor reproducibility and only a very limited understanding of the mechanisms underlying the growth and reproductive processes. The need for a more quantitative technique led to the development of an open, continuous method for cell cultivation, pioneered by Monod (1950).

1.2 Continuous Culture

Although originally conceived by Monod (1950), according to Dawson (1974, 1985d), it may have been the English publication by Herbert et al. (1956) that led to the rapid development of continuous cultivation for studying microbial growth. The basis for the technique is that a constant nutrient environment is provided to actively growing cells by supplying a continuous feed of nutrient solution, with removal of the microbial broth at an equivalent volumetric flow rate. The cells achieve a constant growth rate that is a function of a limiting nutrient concentration, represented by the following empirical equation:

$$\mu = \mu_m(S/(K_s+S)) \qquad (4)$$

where: μ is the specific growth rate $(1/x \cdot dx/dt)$;
: μ_m is the maximum specific growth rate at $2 \cdot K_s$;
: S is the limiting substrate concentration;
: K_S is the saturation constant.

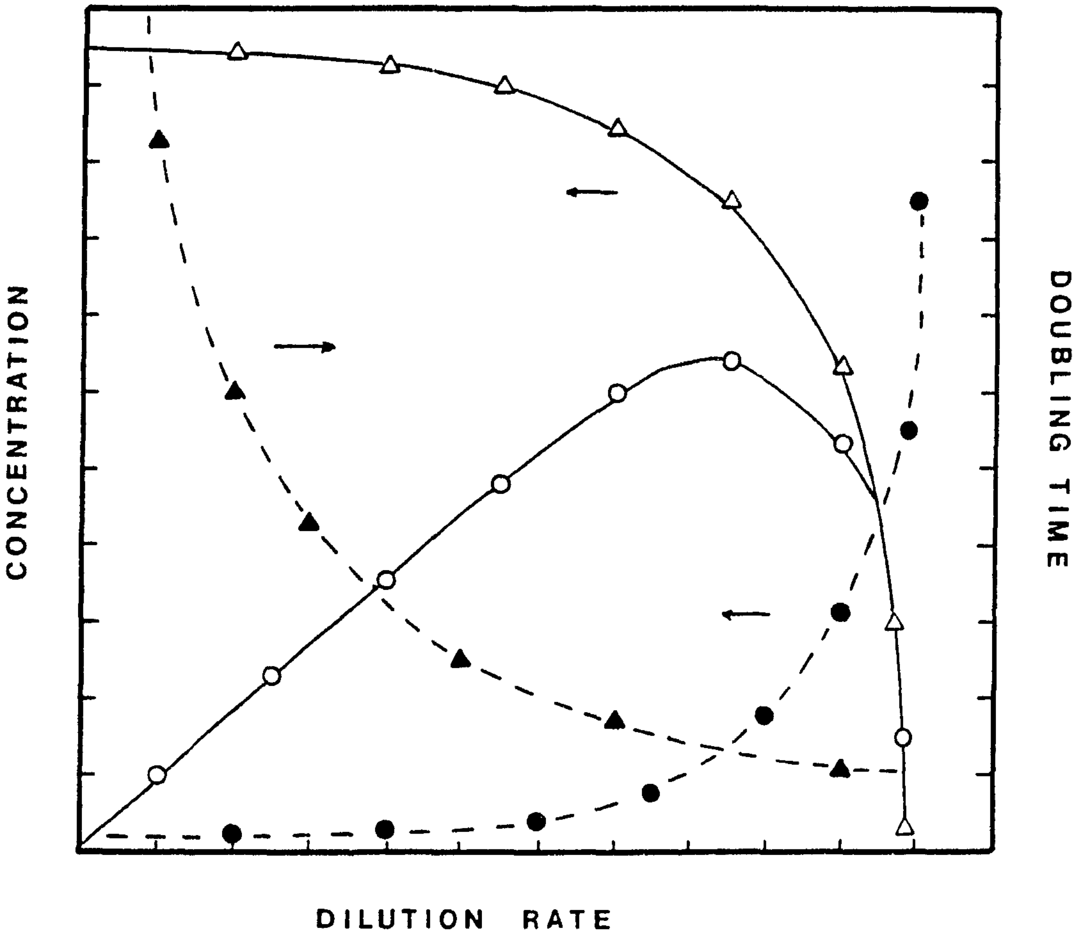
This equation, although empirical, is identical in form to the well-known Michalis-Menton rate equation for enzyme kinetics (Lehninger 1975). This method for continuous culture is considered to be at a steady state if there is no change in biomass concentration. Under this condition, it can be easily shown that the specific growth rate, μ, must equal the volumetric dilution rate, D, where D is the volumetric flow rate divided by the total working liquid volume. The principle of operation is illustrated in Figure 2 (Herbert et al. 1956), showing the effect of changing the dilution rate with a constant substrate concentration in the feed. With a fixed dilution rate the substrate concentration in the feed determines the rate-controlling residual substrate concentration in the fermentor. This corresponds to the condition of when μ and D are equal.

Continuous cultivation of cells using this method clearly has many advantages over the closed batch culture technique. In batch culture only the logarithmic growth phase has a sound theoretical basis (binary fission) and is generally the only reproducible portion of the entire "growth cycle" (Dawson 1985a). By providing a constant nutrient environment for the cells, continuous cultivation was apparently able to eliminate the transient nature of batch culture, achieving a constant growth rate. However, it is important to realize that the Monod equation is empirical and the picture or model of microbial growth is still only relative to the experimental technique employed. Much useful information concerning growth and microbial physiology has been, and will continue to be, obtained using this method. The elimination of the transient

Figure 2

The Effects of Dilution Rate on Continuous Cultivation

Shown is the theoretical relationship at steady state between the bacterial concentration (△), the cell doubling time (▲), the output of bacteria (○) and the substrate concentration in the broth (●) as a function of the dilution rate; from Herbert et al. (1956).


CONCENTRATION
DOUBLING TIME
DILUTION RATE

nature of batch culture allows aspects of growth to be studied systematically, albeit in isolation from the underlying dynamic nature of the cell cycle. The study of dynamic phenomena with a constant input signal is analogous to studying the characteristics of an audio frequency amplifier with a test signal of constant frequency and amplitude. The response of the system to a signal varying in either or both of these parameters (as occurs in music) cannot be predicted because of the non-linear dynamic characteristics of all real components. The same is true for a microbial population due to the dynamic nature of the cell.

A fundamental characteristic or pattern inherent in the concept of an individual cell is its ability to grow and replicate, and this occurs on a cyclical basis with a certain frequency. The characteristics of the cell could, therefore, be revealed by applying a stimulus perceivable within the context of this cycle.

1.3 Cell Synchrony

The obvious method for eliminating or minimizing the inherent averaging effects of cell populations on the underlying cellular behaviour is to use populations of cells synchronized with respect to metabolic functioning. It is generally assumed that metabolic synchrony is synonymous with reproductive synchrony. If the cells divide at the same time, then the metabolic reactions leading up to that division are also likely to be identical. This assumption may not be warranted and all methods for achieving synchrony are not equal in this regard.

The experimental use of cell synchrony dates back to the early 1950s, for example, Tamiya et al (1953) working with Chlorella exposed to light and dark cycles. Early reviews of cell syncrhony were written by James (1966) and Maaloe (1962). An important distinction is made between "synchronous" and "synchronized" cultures of cells. The former is defined as cells dividing simultaneously without the intervention of an external agent while the latter implies the requirement of an external inducing agent (James 1966). This distinction is associated with major differences in experimental technique. Synchronous cultures are produced using passive methods (selection) whereas synchronized cells are produced by active intervention of an environmental stimulus (induction). The issue is further complicated by the use of two broad types of induction: a single event type of blocker, or a cyclic entrainment where the frequency of the inducer corresponds to the cell division cycle.

Induction synchrony can be accomplished with a wide variety of stimuli depending on the specific organism, including: temperature, light, essential nutrients (including gases) and inhibitors. The passive selection technique is usually based on sizing with the assumption of a correlation between size and cell age. The cells can be separated in relation to size using several different techniques, including gradient separation (centrifugation), membrane elution, filtration and electronic sorting (Mitchison 1971). The "washing-off" procedure which takes advantage of selective attachment of parent cells to a solid surface appears to be limited to mammalian cell cultures (Peterson and Anderson 1964).

The proponents of the passive selection techniques criticize induction methods by stating that environmental perturbations shock the culture and, therefore, throw doubt on the "normality" of the ensuing synchronous division and cell cycle events (Edwards 1981). This criticism may in fact be one justification for the induction method. It is clear that cellular metabolism can only be characterized as an interaction between certain inherent capabilities of the cell and its environment. The cell cannot exist outside of a containing and, therefore, influential environment. To maintain that the metabolic processes can be studied in isolation (as they would be "naturally") is unreasonable. Use of selection methods is likely to be inferior to induction since the environment-cell interaction is constantly changing in a non-repeating batch environment. The nature of induction synchrony ensures that the environment-cell interaction is well defined and rigidly controlled such that specific relationships can be revealed. The system is continuous so that both transitory and steady-state situations can be investigated. The fact that induction methods are successful in producing synchronized cultures is a perfect illustration of the profound importance of the temporal nature of the environment during the course of the cell cycle.

1.4 Continuous Phasing

Induction synchrony can be achieved with two different methods. Using a conventional technique for continuous cultivation (chemostat), a growth limiting nutrient is fed continuously into a reactor containing the cell population, while a portion of the broth, containing cells, nutrients and metabolic by-products, is removed at an equal volumetric flow rate. The cells are exposed to a constant concentration of the growth limiting nutrient. Synchronized cell division in a chemostat can be achieved if the limiting nutrient is pulsed into the reactor with a frequency equal to the cell doubling time of the cells (Goodwin 1969a). The alternative is to periodically dilute the culture with an equal volume of fresh nutrients, also with a frequency equal to the cell doubling time. Just prior to dilution, one half of the broth is removed, thus maintaining a constant volume in the reactor and ensuring that each successive generation is grown under an identical nutrient enviornment. This method is referred to as continuous phasing (Dawson 1965, 1969, 1972, Muller and

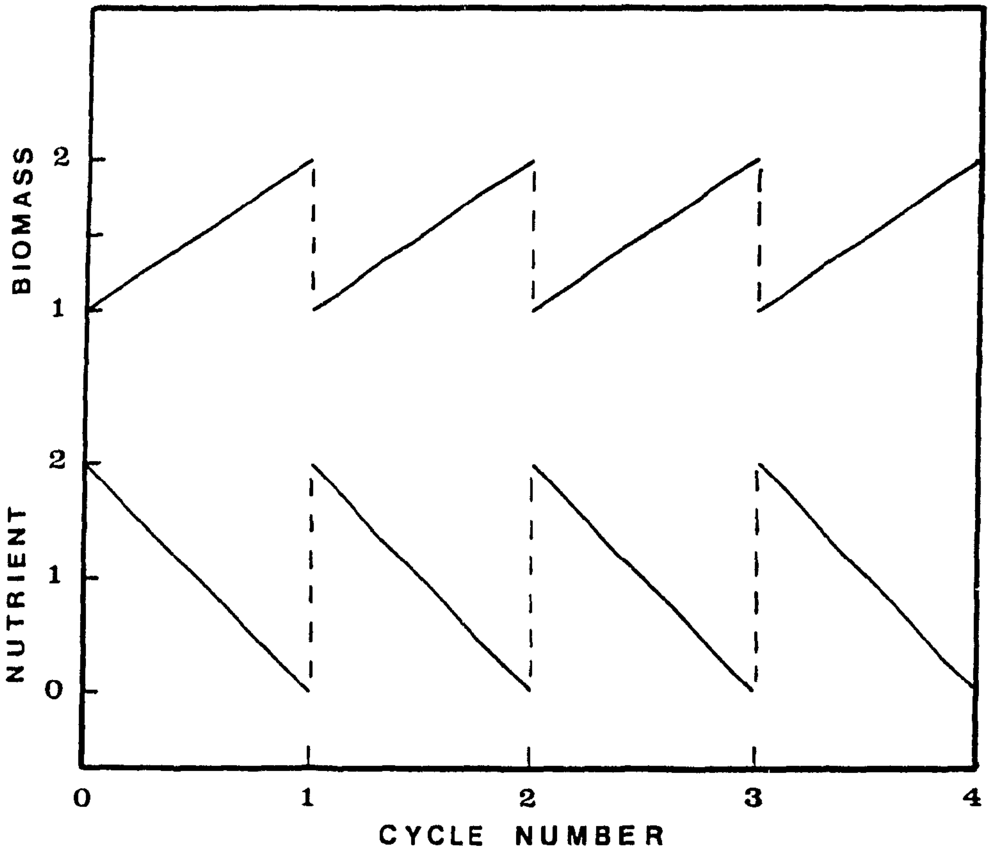
Dawson 1968). Figure 3 illustrates the variation in the concentrations of the limiting nutrient and biomass during continuous phasing. It is important to note that if the biomass at the end of successive cycles is constant, then doubling of the biomass _must_ be occurring during the period of nutrient addition. The advantage of continuous phasing over the pulsed chemostat is that each cycle produces two identical portions of synchronized cells. One portion is retained in the reactor and the second is harvested and made available for second stage or "post cycle" studies (Dawson 1969, 1970); and unlike a pulsed chemostat, the entire nutrient environment is cycled and not just a single nutrient. Cell growth and metabolism have been studied extensively with the use of continuous phasing by Dawson (1971, 1985b), Dawson et al. (1976), Muller and Dawson (1968), Hampton and Dawson (1969) with _Candida_ _utilis_, Anagnostopoulos (1971) with _E. coli_ and by Maruyama et al. (1977) with _Bacillus_ _subtilis_.

Normal operation of a single reactor in a continuous phasing mode results in a quantity of harvested broth equal to one half of the culture volume every complete cycle. Therefore, information can be collected on the conditions in the reactor at the end of one cycle and the beginning of the subsequent cycle but no data is collected during the course of the cycle. It is often very useful to know the pattern of events occurring during the cycle and also after the end of the cycle if fresh nutrients are _not_ added in the so-called

Figure 3

The Principle of Continuous Phasing

Shown are the periodic variations in both the nutrient and the biomass concentrations with arbitrary units. Each nutrient cycle corresponds to one generation of cells.
This is an idealized representation and neither the nutrient or biomass concentrations are likely to change linearly during an individual cycle.


BIOMASS
2
1
NUTRIENT
2
1
0
0
1
2
3
4
CYCLE NUMBER

"post cycle" (Dawson 1969). This information can be readily acquired by operating a second stage reactor concurrently with the primary phasing unit. The inoculum for the second stage is the quantity of broth removed from the primary unit at the end of the previous cycle. Conditions in both reactors should be identical so frequent samples from the second stage can be obtained without being concerned with effects on working volume or risk of contamination in the primary unit. The second stage is normally operated past the normal cycle time to study post cycle events in which the nutrient has been exhausted. It is important to note, however, that this type of post cycle study cannot necessarily be compared to a repetitive type of nutrient starvation that occurs every cycle. In second stage studies the period of starvation is an unexpected occurrence for the population.

Recently, Hjortso (1987) constructed a mathematical model of population growth and tested it to determine under what conditions synchrony would occur as a result of periodic environmental shifts. The model is based on an expression for the age distribution of cells in a microbial culture using a linear partial differential equation which assumes that all cells divide at the same age and division results in two identical cells. The "forcing function" results in periodic changes to the growth rate and associated age at which the cells divide. An example is given where the period of the forcing function is 1.2 and the cell age at division varies between 1.0 and 1.4 in a sawtooth fashion. Because of the interaction between the changing growth rate and the period of the forcing function, the cell age is "attracted" to a particular value, thus resulting in essentially 100% syncrhony in only five cycles. The analytical solution determined the "attractor value" to be 0.3, however, the interpretation of this is unclear since the minimum age is stated to be 1.0. This model is unsatisfactory for two basic reasons: it fails to account for the dynamic nature of the cell cycle itself and makes a serious assumption about the relationship between cell age at division and the periodic forcing function. This relationship is defined by the introduction of a parameter called "adaptability". This parameter is included in an expression for cell age that assumes that the cell growth rate is a weighted average of the growth rate characteristics of all past environments.

$$\text{Transient division age}(t) = \int_{-\alpha}^{t} \text{age}\ (x)\ \alpha e^{-a(t-x)}\, dx \qquad (5)$$

where age (x) is the steady state value of the age at division that is characteristic for the environment at time (x). The adaptability is represented by α. The adaptability factor is considered to be a fixed quantity and the cellular response to the forcing function is related to the history of the environment by a simple exponential decay function obtained from a model for a chemostat (O'Neil and Lyberatos 1986), ignoring the cyclic nature of cellular metabolism.

Goodwin (1963, 1966, 1969a, 1969b) has written extensively on the mechanism of inducing synchrony by periodic nutrient addition. His analysis is based on the observation that the biochemical processes that underly the cell cycle can be considered as feedback control circuits with associated oscillations in the concentrations of both reactants and products (Goodwin 1963). These control circuits operate in a dynamic mode, resulting in a system of internal chemical oscillations that can be more or less strongly coupled (Goodwin 1969b). He reasons that, if the cell is inherently a rhythmic or oscillatory system, then it should be possible to synchronize a population of cells by introducing a periodicity into the environment, thereby entraining the cell. Furthermore, this entrainment could be viewed as a result of "resonance" of the cell cycle with the "oscillatory energy" of the environment. The degree of synchrony and rate at which it occurs within a population would be dependent on the extent of coupling between the metabolic processes and the environmental stimulus (Goodwin 1969b). This concept of oscillator entrainment was described by Engelberg and Hirsch (1966) as a general biological phenomenon encountered in the study of biological rhythms.

Induction synchrony has also been explained by Campbell (1964) by modelling the cell as a simple clock. In such a model the cell cycle is continous with various stages. The time required to complete each stage (and in some cases the entire cycle) is dependent on the environmental conditions. Changing the environment results in a change in the relative times required to complete each stage. It can be shown that periodically shifting the environment will, therefore, result in an increase in division synchrony because of the shift from one stage to another.

The explanation for the success of induction synchrony, and in general the dynamic response of the cells to the environment, must be dependent on the essential nature of the cell growth and reproductive cycle. This nature is expressed or manifest in a way that given the appropriate stimulus it becomes intimately connected with the pattern of the stimulus. More insight into the cell cycle may help explain how this could occur.

1.5 The Cell Cycle

At one time there was a widely accepted paradigm in microbiology of a "normal" cell cycle which reflected some intrinsic property, presumably its genetic capabilities. This is never stated, however, it is implicit in the vast majority of research in microbiology. For example, Edwards (1981) maintains that the cell cycle of prokaryotes is composed of an ordered sequence of events: chromosome replication, septation and cell division, with no mention of growth. In reference to growth he describes "standard" conditions where control is entirely internal with no influence from the environment. The so-called classical cell cycle in eukaryotes is represented by chronological stages of DNA replication: G1, S, G2 and M referring to gap one, DNA synthesis, gap 2 and mitosis. Bacterial cells also have an ordered sequence of events, however, the S period is called C and G2+M is together as a D period (Mandelstam et al. 1982). The variability in G1 is supposed to be responsible for variations in the doubling time (Dawes 1982, Cooper 1984a, 1984b).

The concept of being able to define and observe the behaviour of cells during a "normal" cell cycle is almost ubiquitous in the literature. For example, Mitchison (1971), a proponent of selection synchrony, refers to the cell cycle as being a fixed sequence of events that appear at specific points in a normal cycle but can appear at different times or in a different order if the cycle is "distorted". Necas (1984) refers to the cell as a "reproductive automation" and explains how the reproductive cycle is constant and will depend only on the structure of the replicating system. However, he qualifies his hypothesis by stating that the ideal generation time can only occur when the conditions are "optimal" (for maximum growth rate) and these ideal conditions are unlikely to be realized. It is clear that they can never be realized because it is impossible to know if they ever have been realized. To state that the maximum growth rate is the optimal growth rate is in itself a value judgement that may have no relationship to a so-called normal cell cycle. We are left with the epistomological fact of having to define the cell cycle in terms of the environment in which it is expressed.

This concept of an intrinsic pattern in cell reproduction has undoubtedly helped justify the widespread acceptance of both batch and chemostat methods for cell culturing. Since neither method permits observation of the cell cycle, it is usually assumed that variations in the cycle either are not important or do not exist. Dawson (1985) emphasizes that continuous phasing reveals a cell cycle that is inexorably connected with its environment and cannot be described in isolation.

It is clear that growth and reproduction are related but not synonymous because of the apparent uncoupling that can occur when DNA replication is blocked (Prescott 1964, Mitchison 1971), although Cooper (1984a, 1984b) believes that growth is essentially a characteristic of the G1 phase and is, therefore, inherently part of the DNA replication sequence. When a population of cells is growing and cell number is changing, the separation between growth and reproduction becomes semantic. Growth of the cell can be defined as part of the process of reproduction or reproduction of the cell as an inevitable consequence of growth. However, in both cases it should be defined from a dynamic perspective as a continuously evolving process, not as a fixed sequence of events frozen in time without consideration for their historical context.

Considering the cell as a dynamic process is compatible with observed oscillations in the biochemical processes underlying the cell cycle and also the macromolecular behaviour of populations of cells subjected to various environmental stimuli. To quote D'Arcy Thompson (from Mendelson 1982) "From the moment that we enter on a dynamic conception of the cell, we perceive that the old debates were vain as to what visible portions of the cell were active or passive, living or non-living. For the manifestations of force can only be due to the interaction of the various parts, to the transference of energy from one to another." Over the last thirty years there has been an exponential increase in the number of publications dealing with biological phenomena from the dynamic perspective. A review by Winfree (1980) contains at least 1100 references with 140 from 1979 alone. The volume of literature precludes the possibility of covering all aspects of biological oscillations. However, it is clear that similar approaches to understanding biological systems can be applied on many levels: from biochemical reactions that occur in nanoseconds to population cycles spanning many years (Lloyd et al. 1982).

The most common analogy used in reference to the cell cycle is that of the clock (Mendelson 1982, Cooper 1984a, 1984b, Lloyd and Edwards 1984, Poole 1984, Klevecz 1984).

The clock is essentially a device that exhibits a repeating pattern over timetabe. This pattern can be considered as either continuous or composed of arbitrary incremental steps. In this respect a swinging pendulum is identical to rotating hands, although the physical nature of the pattern produced by a pendulum is fundamentally different than rotating hands. The pendulum is said to oscillate because the direction of movement at some point in the cycle changes abruptly, not continuously. The unidirectional aspect of a rotating mechanism gives the impression that time is like a vector from past to future, whereas the pendulum gives the impression of eternal repetition, an oscillation between two states in two directions.

Oscillatory stimuli are required for perception and are considered to be the basis for biological control systems (Goodwin 1963, Sel'kov 1968, Higgins 1964, Mackey and Glass 1977, Chay 1981, Pavlidis 1973). Control requires organization, leading to the application of cybernetics (Apter and Wolpert 1965, Calow 1976, Majernik 1970) and synergetics (Frohilich 1977, Haken 1987) in biology. When considering the cell cycle, instead of describing motion or spatial relationships, it seems more appropriate to describe it as a repeating transformation of raw materials into a fully integrated functioning whole. Therefore, the cell cycle is more oscillatory than rotational. The cell cycle has been described as an oscillator by Shymko et al. (1984), Klevecz

(1984) and particularly Gilbert (1968, 1978, 1984) who has suggested that each species of cell has its own pattern of temporal organization that reflects its own unique pattern of metabolism.

Gilbert (1968) maintains that cells have an overall dynamic state that is periodic as a result of the coordination of the underlying rhythmic processes. This coordination can be explained by a widespread phenomenon referred to as oscillator entrainment or coupling. Oscillations of similar frequency interact such that both frequency and phase locking can occur (Gilbert 1984, Ermentrout 1981). Oscillator coupling can explain the mechanism for temporal organization in the cell and also the interactions between cellular metabolism and periodic environmental stimuli as occurs with circadian rhythms (Pavlidis 1973, Winfree 1980, Klevecz 1984).

1.6 Coupled Oscillators

One of the first suggestions that cellular metabolism could be considered as a system of coupled oscillators came from Goodwin (1966), based on the theory that natural oscillations occur as a result of feedback repression circuits (Goodwin 1963, Masters and Donachie 1966). Coupled oscillators had previously been used to explain the apparent entrainment of circadian rhythms in some organisms subjected to an external stimulus of light and dark cycles (Pittendrigh and Minis 1964). Goodwin (1966) proposed that enzyme synthesis could be entrained by periodic bursts of mRNA synthesis, thereby preserving the linear relations of the genetic code in the phase relations of the enzyme oscillations. Pavlidis (1973) has provided an extensive mathematical analysis of various forms of coupled oscillators as encountered in biological systems.

In a population of cells, past and future generations are related as a result of three factors: they have a common genetic make-up; they have a common metabolic ancestry to the extent that each parent generation is subjected to the same environmental conditions during growth; the environment which each daughter generation encounters has been affected by the parent cell generation. Therefore, it would be expected that if growth of the population was not based on a cyclical process, each generation of cells would display metabolic differences from the parents. This would be true in both batch cultures and chemostats, although in the latter case the constant nutrient feed would be more effective in preventing excessive metabolic deviations due to the self-compensating nature of the method.

A conventional batch growth of cells over many generations cannot reveal any real relationship between the environment and the individual generations but instead reveals transient population responses. It is not a repetitive phenomenon nor is an equilibrium achieved. A non-transient response can be obtained by coupling the cell generations together in a cycle of repetitive interdependence. An external oscillation of a growth controlling nutrient can act as a coupling agent, thereby creating a "hypercycle" as described by Eigen and Schuster (1977, 1978). A hypercycle can be represented by a cyclical coupling of related but self-contained cycles. A hypercycle has a higher level of organization than the individual component cycles and therefore a higher information content. The information content of the hypercycle is composed of information from each of the component cycles plus their coupling or functional linkages. Hypercyclic organization is a uniquely stable state that results from a competitive advantag over other more poorly integrated component cycles (Eigen and Schuster 1977).

The principle of hypercyclic organization will likely only be valid over a limited range of periods of the environmental oscillation. The range of effectiveness will be dependent on the period of oscillation in the system being acted upon. For example, if the environmental oscillation acts on the entire cell growth and reproductive cycle, then the period of the oscillation must span at least two cell generations, otherwise no coupling is possible. If, on the other hand, the environmental stimulus is perceived on the metabolic level, which has its own inherent periodicity, then the period of the stimulus should be less than or equal to a single cell generation.

When the environment stimulus is perceived directly on the metabolic level, it is likely that the organizing (or entrainment) effect will be most pronounced if the period of the stimulus corresponds precisely to the cell generation time. This is because the cell generation time spans the entire history of the growth and reproductive processes, at the conclusion of which a distinct discontinuity occurs, the time of cell division. This would account for the success of producing a synchronous cell culture by the periodic addition of a limiting nutrient. Not only do the individual cells tend to react simultaneously to the environmental cycle, but the metabolic processes revealed in this way reflect an organization that is truly indicative of the dynamic interaction between the genetic capabilities of the cell and the environment.

1.7 Bacillus subtilis ATCC 21332

Many biological compounds exhibit surface activity. Most are lipids composed of a lipophilic saturated or unsaturated hydrocarbon tail attached to a polar head group. The hydrophilic head group may be a simple carboxylate or hydroxyl function or a more complex mixture of carbohydrates, phosphates or amino acids. Surfactants of microbial origin have been extensively reviewed by Zajic and Seffens (1984), Parkinson (1985), Rosenberg (1986) and Cooper (1986).

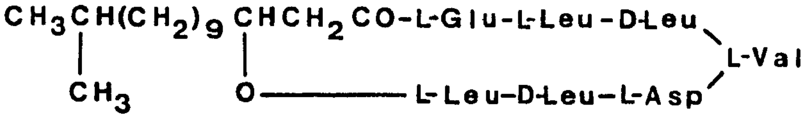
Some strains of Bacillus subtilis have been shown to produce significant quantities of a lipopeptide with potent surface activity, lowering the surface tension of water from 72 to 27 mN/m. This compound was first reported and named "surfactin" by Arima et al. (1968) in a study that described certain of its effects including inhibition of blood clot formation, inhibition of protein denaturation and the lysis of both gram positive and gram negative bacteria with the leakage of cell contents. However, they were unable to determine the physiological role of surfactin in the producing bacteria. Subsequently Kakinuma et al. (1969a, 1969b) elucidated the full structure as being a cyclic lipopeptide containing a carboxylic acid (3-hydroxy-13-methyl tetradecanoic acid) and seven amino acids composed of L-aspartic, L-glutamic, L-valine, L-leucine and D-leucine in the relative portions of 1:1:1:2:2 respectively. The L-glutamic acid is N-bonded to the carboxylate of the fatty acid and L-leucine is bonded to the 3-hydroxyl function to form a lactone ring (Figure 4).

Bernheimer and Avigad (1970) described a substance responsible for the lysis of red blood cells by cultures of Bacillus subtilis. This substance originally designed as "subtilysin" was found to have identical properties to surfactin. Haemolysis was activated by magnesium, manganese and calcium ions and inhibited by sera containing globulins

Figure 4

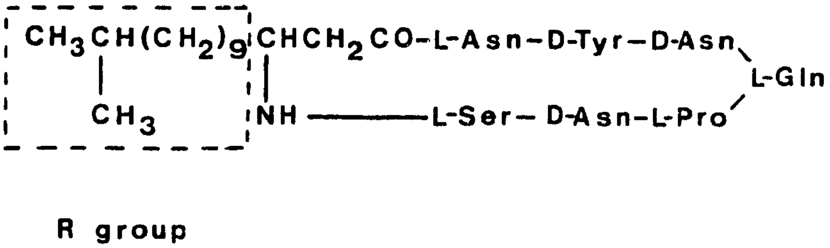
The Structures of Surfactin and Iturin A

Shown are the similar structures of extracellular lipopeptides produced by different strains of B. subtilis. The R group can vary in the iturins.



$$\mathrm{CH_3\underset{\displaystyle CH_3}{CH}(CH_2)_9\underset{\displaystyle O}{CH}CH_2CO\text{-}L\text{-}Glu\text{-}L\text{-}Leu\text{-}D\text{-}Leu\text{-}L\text{-}Val\text{-}L\text{-}Asp\text{-}D\text{-}Leu\text{-}L\text{-}Leu\text{-}O}$$

Surfactin



$$\mathrm{\boxed{CH_3\underset{\displaystyle CH_3}{CH}(CH_2)_9}\underset{\displaystyle NH}{CH}CH_2CO\text{-}L\text{-}Asn\text{-}D\text{-}Tyr\text{-}D\text{-}Asn\text{-}L\text{-}Gln\text{-}L\text{-}Pro\text{-}D\text{-}Asn\text{-}L\text{-}Ser\text{-}NH}$$

Iturin A

or low concentrations of phospholipids. Surfactin possessed antibiotic properties, lysing protoplasts and spheroplasts from several bacterial species, and shared some properties with the other microbial cytolytic agents staphylcoccal α-toxin and streptolysin S. Recently, bench-scale production of surfactin has been described by Cooper et al. (1981), Vater (1986) and Sheppard and Mulligan (1987), although there is also growing interest in the genetic factors (Nakano et al. 1988).

The commercial interest in biosurfactants continues to increase. Reiling et al. (1986) and Guerra-Santos et al. (1986) have reported on the pilot plant production of a rhamnolipid by Pseudomonas aeruginosa and Batelle in the U.S.A. claim to be near to commercializing a biosurfactant for the over $1 billion per year industrial market (Bioprocessing Technology Vol. 9, 7, 1987).

Biosurfactants have several advantages over most conventional synthetic surfactants from hydrocarbons including: low toxicity, high specificity and high activity. The key to successful commercialization is to both minimize production costs and identify specific applications for which the unique properties of the biosurfactant can be exploited. If the natural physiological role for the compound can be determined, this will help to both optimize the production process and provide insight into potential applications.

Optimal production requires that the nutrient environment be rigorously controlled. The nutrient environment includes both dissovled solids and gases. Oxygen is an essential nutrient for B. subtilis and, therefore, providing adequate capability for gas-liquid transfer is an important process consideration.

1.8 Oxygen Transfer

Oxygen can be considered as an essential nutrient for an aerobic organism such as B. subtilis. However, unlike most other nutrients, oxygen has very low solubility in water, 1.16 x 10^{-3} moles per liter at 30°C, and is consumed at a rate that can deplete the broth in seconds (Bailey and Ollis 1977). Therefore, oxygen must be supplied continuously to the cell culture.

The effects of surfactants on gas transfer into liquids have been studied extensively, however, the conclusions are not always consistent, probably due to the complexity of the phenomena and the difficulty of accurately measuring rates of gas transfer.

It is generally accepted that surfactants can affect gas absorption by changing the interfacial film characteristics in two ways. The first effect, which is much more important with insoluble surfactants (Springer and Pigford 1970, Burnett and Himmelblau 1970), but has also been measured in the presence of soluble surfactants (Caskey and Barlage 1972, Cullen and Davidson 1956), is an increased diffusional resistance. Princen et al. (1967) claim that diffusion actually takes place through aqueous pores between the surfactant molecules. However, of greater importance are likely to be the effects on hydrodynamics, both at the scale of the interfacial film (Burnett and Himmelblau 1970, Springer and Pigford 1970, Lee et al. 1980, Goodridge and Robb 1965, Andrews et al. 1988) and the interfacial area in the reactor (Benedek and Heideger 1971). Although these effects are clearly related, they are sometimes treated as separate phenomena (Eckenfelder and Barnhart 1961). The prediction of the oxygen transfer rate at various concentrations of surfactants in different systems is very difficult due to these several factors which can, in some cases, be self compensating. For example, at low surfactant concentrations the effects on film resistance may predominate, thereby lowering the rate. At higher concentrations the effects on interfacial area may be significant enough to increase the rate (Eckenfelder and Barnhart 1961, Benedek and Heideger 1971). Other factors such as rate of shear (Prins 1976) and presence of impurities (Cullen and Davidson 1956) should also be considered but make prediction difficult. In any event, it is necessary to consider oxygen transfer as a key parameter in process design and scale-up, especially in the presence of surfactants.

1.9 Objectives

Optimized production of a metabolite can only be accomplished when the relationships between the environment in which the cell is grown and the cellular metabolism which affects metabolite production are understood. Only then can the environment be controlled to produce the desired cellular performance. B. subtilis is known to produce several compounds that exhibit antibiotic activity, for example Iturin A and Bacillomycin L (Besson et al. 1987, Chevanet et al. 1986) which like surfactin are cyclic lipopeptides (Figure 4). However, cell culture studies almost always use an asynchronous batch population in which the growth limiting nutrient is not even known. The results of such studies summarily state which nutrients increase production and which decrease production under a certain set of conditions. The number of possible nutrient permutations is so large and the metabolic processes so complex (apparently) that causal connections are almost never elucidated. Therefore, there is no way of knowing whether the environment is optimal, or even designing an approach to optimization. Improvements in production are largely a result of trial and error experimentation which may or may not be successful. The economic feasibility for production should not have to be based on the chance occurrence of providing an adequate environment for the cells.

This project is designed to develop a technique for cell culturing that reveals real causal connections between the environment and cellular metabolism as reflected by the growth of B. subtilis ATCC 21332 and the production of surfactin. Once these causal connections can be made, then the engineer and microbiologist can proceed to build a fundamental model with each experiment increasing the scope of understanding in a unified manner. The complexity of the underlying metabolism that is involved with a particular process (for example surfactin production) will determine the scope that the model must encompass to permit complete process optimization. Although a full understanding may not be practically attained, at least the technique will ensure a rational progression towards this ideal.

The specific experimental plan includes:

i) Characterization of the growth of B. subtilis ATCC 21332 and the production of surfactin using the conventional batch culture technique. Maximum specific growth rates obtained during the logarithmic growth phase in a specific medium will be used to select appropriate periods for nutrient cycling during continuous phasing.

ii) Continuous phasing of B. subtilis will be employed using a variety of imposed periods of nutrient cycling. Second stage studies will be performed to investigate the production of surfactin as it relates to the cell cycle.

iii) A computerized feedback control system based on the concentration of dissolved oxygen will be developed in order to increase the stability of the system during continuous phasing, and to provide precise control over nutrient starvation.

iv) Procedures will be tested and refined for the purification and identification of the extracelluar product surfactin.

v) The effects of surfactants, and particularly surfactin, on the oxygen transfer characteristics of the cyclone column reactor will be investigated. This is to ensure that adequate oxygen is available for growth of the cells and to estimate the maximum biomass concentration that can be sustained in the presence of surfactin.

vi) A technique for investigating the response of the cell population to changes in the medium composition will be developed and the production rate of surfactin estimated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Biomass Concentration

The concentration of biomass in the fermentation broth was determined either directly by dry weight or indirectly by optical density.

Optical density measurements were made using a dual beam scanning UV-visible spectrophotometer (Varian Model DMS200) set at a fixed wavelength of 600 nm. Distilled water was placed in the reference cell and about 3 mL of broth were placed in the sample cell. After about 30 seconds of equilibration, the optical density was recorded, plus or minus 0.005 absorbance units.

Dry weight was determined by centrifuging approximately 125 mL of broth at 6000 RPM for 20 minutes in a Sorval refrigerated centrifuge Model RC-5 equipped with a GSA rotor. The supernatant was removed and the cells were washed with about 100 mL of distilled water and then recentrifuged. After removing the supernatant the cells were transferred with a small volume of distilled water to pre-weighed aluminum dishes. The dishes were then placed in a convection drying oven (Fisher Model 126G) at 105°C and dried until a constant weight. Weights were determined using an analytical balance (Mettler Model AE 160) with an accuracy of 0.1 mg.

There are potential sources of error using either of these methods and often there is a poor correlation between optical density and dry weight. The optical density is particularly sensitive to the length of the delay between obtaining the sample and performing the analysis. It was determined that a refrigerated sample could decrease its optical density by as much as 40% over 24 hours. This could be a result of cell lysis, clumping or changes in the cell's surface characteristics. Dry weight seemed to be less affected by storage time, the largest source of error was likely due to inefficient washing of the cells due to poor cell compaction while centrifuging. However, dry weight analysis requires relatively large sample volumes compared to optical density. Therefore, biomass was generally determined by dry weight during continuous phasing when large sample volumes were available and optical density was used during second stage studies (as described in Section 1.4) and for batch fermentations. The correlation between the two measurements, although somewhat variable, was about 0.3 grams of cells per liter per absorbance unit.

2.2 Viable Cell Count

It was considered important to test for numbers of viable cells so that the degree of doubling synchrony could be determined and also to monitor any changes in cell number that might correlate with changes to the environment or surfactant production. The method selected was a plating procedure essentially as described by Postgate (1969). Although there are potentially many sources of error (Postgate 1967), it was considered to be the best method available.

After a number of trials good reproducibility (less than 10% difference between duplicate plates) was achieved with the following procedure. Depending on the expected cell number, either 5 μl or 10 μl (or both) of fresh broth was measured with a Gilson micro-pipette and transferred into a sterile 150 mL glass bottle containing 100 mL of sterile mineral salts and glucose medium. The bottle was vigorously shaken about 25 times and then 1 mL was transferred with a sterile 1 mL pipette into a sterile test tube containing 9 mL of identical medium. The test tube was then vortexed at high speed for 5-10 seconds. From the test tube two 0.1 mL aliquots were obtained with a 1 mL pipette and each spread with a glass rod over a 9 cm nutrient agar petri plate. The plates were incubated at 30°C for 24 hours and then the individual colonies were counted. The number of colonies were converted to viable cell count in the broth by multiplying by the dilution factor. For example, starting with 5 μl of broth and finishing with 50 colonies per plate would result in a total viable cell count of:

$$50 \times (100 \times 1000 \times 10 \times 10)/5 = 100 \times 10^6 \text{ per mL.}$$

2.3 Surfactin Concentration

Surface tension is a very sensitive measurement for monitoring the presence of surface active agents in an aqueous sample. The surface tension of a pure solution will be linearly correlated to the concentration of the active species until the critical micelle concentration is reached (Rosen 1978), after which there will be little change as any additional surfactant will be present in a micellar form. However, in non-pure systems (any real system) impurities may significantly affect the interfacial behaviour of surfactants and also contribute to changes in surface tension. Therefore, surface tension data from non-pure solutions must be considered semi-quantitative. In these studies surface tension has been used as a general indication of the presence of surfactants in the broth and is valid for comparing relative changes. Fortunately, surfactin is a particularly potent surfactant capable of lowering the surface tension down to 27 mN/m, below where the effects of most impurities would be important.

The surface tension measurements were performed with a Fisher Autotensiomat (Model 215) connected to a Fisher Recorder Series 5000. The tensiometer functions by lowering a stage on which a container holding the sample has been placed, with a platinum-iridium ring immersed below the air-water interface. As the stage is lowered (1.25 cm/min) the ring is pulled through the interface, thereby pulling down on a strain gauge. The force, as measured by the strain gauge, is then converted to units of surface tension (mN/m). The tensiometer is calibrated with a one gram weight which corresponds to 82 mN/m. This method is referred to as a the du Nouy method. The ring is flamed between samples to remove any residual material and the tests are performed 3 or 4 times or until constant readings are obtained. The samples are allowed to equilibrate with the air at room temperature for about 30 minutes prior to testing. Experience has shown that the results are usually reproducible within plus or minus one mN/m. All samples were cell-free.

Surface tension measurements can give semi-quantitative information on surfactant concentration by calculating the reciprocal of the critical micelle concentration (Cooper et al. 1981). This procedure was slightly modified by Sheppard and Mulligan (1987) and is described in detail in Appendix A. More quantitative surfactin yields were obtained by recovering and purifying the crude product to obtain a weight of surfactin per unit of broth. The purity of the final product was evaluated with an amino acid analysis.

Several modifications of the technique described by Cooper et al. (1981) for the recovery of surfactin were tried. In all cases the cells were first removed from the broth by centrifugation. The pH of the broth was then lowered to 2 by adding concentrated hydrochloric acid. This resulted in precipitation of the proteins and the lipopeptide surfactin. At this point, addition of an equal volume of chloroform to the broth would result in a separation of the lipids (including surfactin) from the broth and precipitated proteins. However, this method required considerable quantities of chloroform because of the tendency of an emulsion to form at the interface of the aqueous and chloroform layers. Lipids other than surfactin would also be recovered with this method. To overcome these difficultues, the acid precipitate was removed from the broth by centrifugation for 20 minutes at 10,000 RPM in 125 mL pyrex tubes using a GSA rotor (Sorval). The broth was discarded and the solids removed from the tubes with a small volume (about 10 ml per tube) of distilled water. This small volume was then extracted with chloroform. Lipids other than surfactin would no longer be present and the increased ratio of chloroform to water prevented an emulsion from forming. The recovered chloroform layer was subsequently evaporated at room temperature and the remaining solids weighed to calculate the final yield. A portion of the solids could then be redissolved in chloroform for further analysis by thin layer chromotography (TLC) or, alternatively, hydrolysed for an amino acid anlaysis. The only problem encountered with this modified technique was the difficulty in redissolving the final product in water. Even after adjusting the pH to 9 or 10 with 0.5N sodium hydroxide, the surfactin seemed to be essentially insoluble, unless solutions were prepared very dilute (above the critical micelle concentration). However, this problem was largely overcome by another minor modification to the procedure. Before the recovered acid precipitate was extracted with chloroform, the solids were air dried at room temperature. The dried solids were then mixed with chloroform with essentially no water present. The chloroform-insoluble material was removed by filtration (5.5 cm, Reeve Angel 934 AH glass fiber filters) and the chloroform evaporated at room temperature. The surfactin recovered with this new technique was found to be more readily soluble in slightly basic water.

2.4 Ammonia Nitrogen Concentration

Most of the experiments were performed using nitrogen as the growth limiting nutrient. It was therefore important to be able to accurately determine the concentration of nitrogen in the broth during batch growth and second stage studies where specific cell cycles were examined. Routine operation of the continuous phasing unit did not require nitrogen analyses because the nitrogen was always exhausted by the end of the cycle.

The nitrogen analyses were performed as described by Conway (1957) and is called the Conway Microdiffusion Analysis. Special microdiffusion dishes are required with two concentric chambers. One mL of a 1% boric acid solution containing 1% mixed indicator (0.033% bromcresol green plus 0.066% methyl red in alcohol) was added to the inner chamber and then 0.5 mL of saturated Na_2CO_3 (alternatively K_2CO_3) to the outer chamber. These volumes need only be approximate. A Gilson micropipette was then used to transfer 0.5 mL (+ or - 0.01 mL) of the cell-free sample into the outer chamber without mixing with the Na_2CO_3. The cover was placed on the disk and sealed air tight with silicone vacuum grease. The dish was then slowly rotated to mix the sample with the Na_2CO_3, taking care not to spill into the inner chamber. The mildly basic conditions resulted in a release of ammonia gas from the residual ammonium in the sample. The ammonia was absorbed by the pink boric acid solution resulting in a colour change to green. After leaving the dishes overnight, the boric acid solution in the inner chamber was titrated with 0.02 N HCl using a 2 mL (±0.001 mL) Gilmont microsyringe until the colour returned to the original pink. The volume of acid required was directly proportional to the concentration of ammonium ions in the original sample. The procedure was calibrated using a stock solution of $(NH_4)_2HPO_4$ and was found to be accurate to within about 0.1 mg per liter with duplicate dishes. Ammonia nitrogen concentrations in the broth ranged between zero and 22 mg/L when using a 0.002 molar $(NH_4)_2HPO_4$ in the medium. The calibration factor was determined each time new solutions were prepared but was typically 3.09 mL of

0.02 normal HCl per mg nitrogen.

2.5 Dissolved Oxygen Concentration

The concentration of dissolved oxygen in the fermentation broth, medium or various surfactant solutions was measured using an Ingold Model IL 531 polarographic oxygen sensor connected to a Pegasus multi-meter. The multi-meter provided the amplifier and a 4-20 mA output that was measured by connecting a Fisher Model chart recorder across a 47 ohm resistor. After calibration, the dissolved oxygen concentration was proportional to a 0-0.8 volt reading on the chart recorder. The data acquisition unit (HP 3421A) could also be used to convert the analogue output to a digital signal for monitoring by the computer (HP87XM).

The IL 531 contains two electrodes suspended in an electrolyte and isolated from the sample by a stainless steel reinforced silicone membrane. Oxygen diffuses through the membrane where it is reduced at the platinum cathode to hydroxyl ions. A current flows between the cathode and anode (silver/silver chloride) that is proportional to the concentration of oxygen in the sample. The probe can be sterilized by heating at 120°C for 20 minutes and has a built-in thermistor for temperature compensation between 15°C and 45°C.

The manufacturer claims a response time of 98% in 45 seconds. This was experimentally verified by placing the probe in a rapidly agitated beaker of water in which air was stripped by a constant flow of nitrogen. After equilibration to zero percent saturation the probe was transferred (in about one second) to a beaker which contained water saturated with air and also rapidly mixed. This was repeated five times in each direction (gassing-in and gassing-out) and the average response time was determined to be 90% in 18.7 seconds.

The probe was mounted in a specially designed and fabricated glass fitting placed directly below the outlet from the cyclone column. The recirculating pump sucked the broth past the probe at high velocity (0.46 meters per second), ensuring no stagnant layer or dead spaces around the membrane.

2.6 Thin Layer Chromatography

It was desirable to confirm the presence of surfactin and determine if more than one extracellular lipopeptide was present. Thin layer chromatography (TLC) was previously used by Cooper et al. (1981) to isolate surfactin from *B. subtilis* broth and also by Besson and Michel (1987) for the separation of the iturin lipopeptide antibiotics from *B. subtilis*. The extracellular products were recovered from the broth and purified as described in Section 2.3

Thin layer chromotography was performed using silica gel plates with a $CaSO_4$ binder (Fisher Scientific) pre-dried at

105°C. The solvents were either a mixture of chloroform, methanol and water (65/25/4 by volume), chloroform, methanol and 28% ammonium hydroxide (65/25/4 by volume) or chloroform, methanol and acetic acid (65/25/4 by volume). Typically 0.2 mL of a 4 mL chloroform solution of the recovered products were spotted on the plate about 2 cm from the bottom. The plate was placed in a tank containing 188 mL of equilibrated solvent mixture and saturated filter paper lining the sides. The plate was removed from the tank after the solvent front reached about 2 cm from the top of the plate. The spots were developed with one of a variety of methods as described by Kates (1972), although development in a chamber saturated with iodine vapour was preferred. After development, the spots were circled and R_f values measured relative to the solvent front.

2.7 Manganese Concentration

It has been shown by Cooper et al. (1981) that certain divalent cations, in particular manganese and iron, affect the growth and/or surfactin production by B. subtilis ATCC 21332. Some of the experiments in this study employed periodic changes in the manganese concentration in the cell broth. Analysis of the cell-free broth for manganese was performed by measuring atomic absorption using a Smith-Hieftje 11 analyser (Thermo Jarrel Corporation). The lamp current was

5.0 mA and detection was at 279.5 nm with a 0.5 nm bandwidth. A standard nebulizer (121645 L-A) was used, adjusted to give an aspiration rate of about 2 mL/min with gas flows of 1.4 x 10^{-3} m^3/min of acetylene and 5.7 x 10^{-3} m^3/min of air.

Calibration curves were prepared just prior to performing the analysis each day. A typical calibration curve is shown in Figure 5. The absorbance as a function of manganese concentration was linear up to a manganese concentration of about 5 x 10^{-5} molar, corresponding to an absorbance of about 0.65. Samples were analyzed by obtaining absorbance measurements every second for five consecutive seconds while continuously aspirating. The Smith-Hieftje 11 automatically averaged the readings and calculated the relative standard deviation (RSD). If the RSD was 1% or less, then the results were accepted. The absorbance was converted to manganese concentration with the calibration curve.

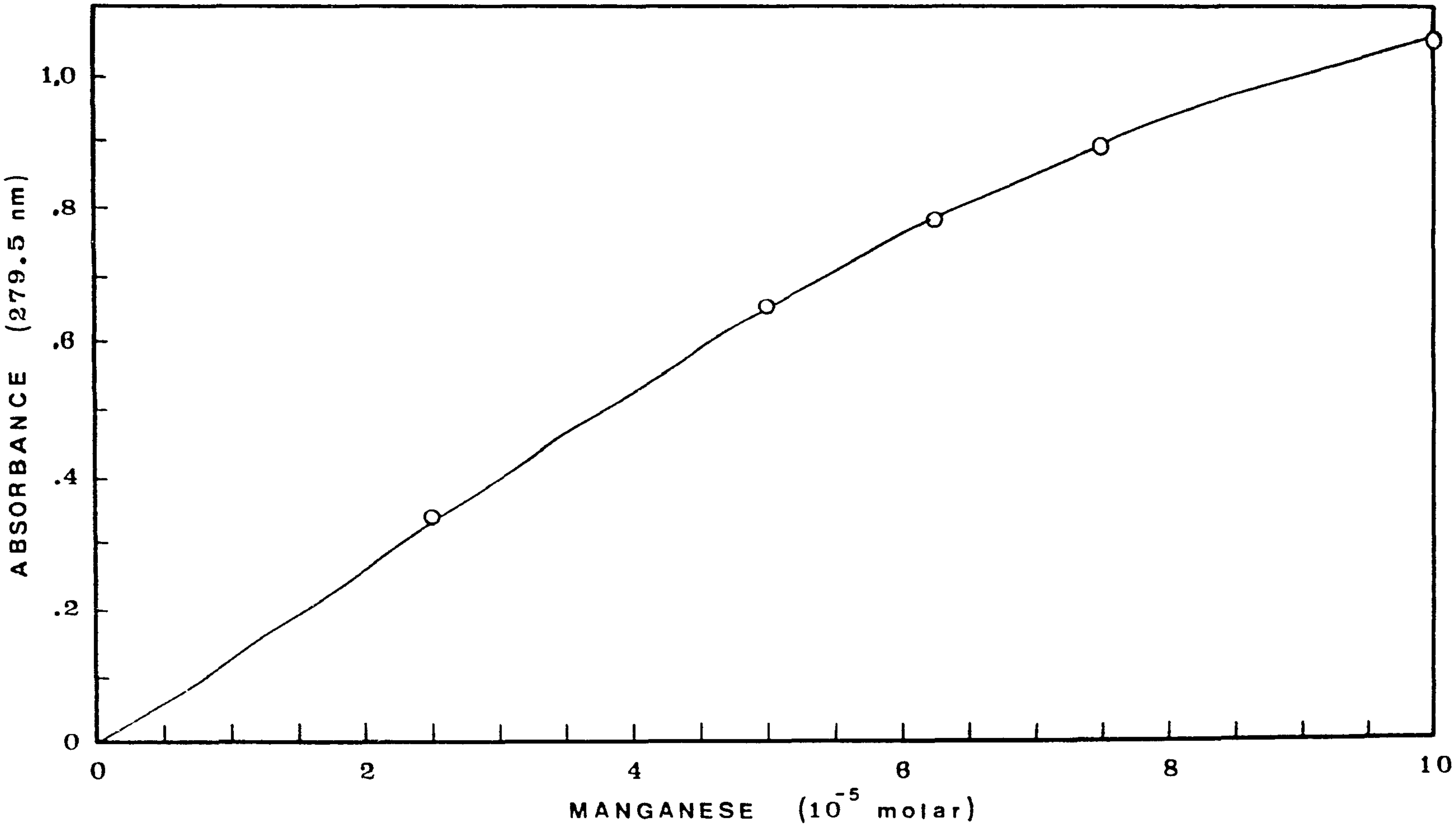
2.8 Amino Acid Analysis

Surfactin contains a peptide composed of four amino acids - leucine, aspartic acid, glutamic acid and valine (Kakinuma et al. 1969a, 1969b). They are found in the ratio of 4:1:1:1, leucine being the most abundant. Analysis of the product recovered from the broth with respect to its amino acid complement, will provide an estimate of the purity. Amino acids, other than these four, will likely originate from other

Figure 5

Calibration Curve for Determination of Manganese Concentration by Atomic Absorption

Shown is a typical calibration curve based on serial dilutions of a stock solution of 1 x 10^{-4} molar $MnSO_4 \cdot H_2O$.


ABSORBANCE (279.5 nm)
1,0
.8
.6
.4
.2
0
0
2
4
6
8
10
MANGANESE $(10^{-5}$ molar)

extracellular proteins or products of cell lysis that have contaminated the surfactin.

The amino acid analysis was performed with a Beckman System 6300 High Performance Analyser. The samples of product were recovered from the broth as described in Section 2.3 and the dried solids hydrolyzed with 6 N constant volume HCl in a Waters Pico-Tag Work Station for 2.5 hours. The samples were then diluted to a volume of 200 μl and 50 μl were injected into the analyser. The results are reported as nanomoles of each amino acid present.

2.9 Medium Preparation

The composition of the medium was based on the recipe published by Cooper et al. (1981), with several variations. The contents of these media are presented in Table 1.

The most important aspect of medium composition is the knowledge of all the components and which of these components is the growth limiting nutrient. This is necessary in order to permit interpretation of the metabolic responses to changes in either the temporal or chemical nature of the cell's environment. In these studies the growth limiting nutrient is defined as the nutrient that becomes completely exhausted in the medium, thereby stopping further growth of the organism. Obviously metabolic processes continue after growth has stopped and these processes can be very important for

Table 1

Medium Composition

Ingredient	Molarity		
	Cooper's*	C-limited	N-limited
$Na_2 \cdot EDTA \cdot 2H_2O$	4×10^{-6}	1×10^{-3}	1×10^{-3}
$MnSO_4 \cdot H_2O$**	0	5×10^{-5}	3×10^{-4}
$FeSO_4 \cdot 7H_2O$**	4×10^{-6}	1×10^{-4}	1×10^{-4}
$MgSO_4 \cdot 7H_2O$	8×10^{-4}	8×10^{-4}	8×10^{-4}
$CaCl_2 \cdot 2H_2O$	7×10^{-6}	7×10^{-6}	7×10^{-6}
Na_2HPO_4	4×10^{-2}	4×10^{-2}	4×10^{-2}
KH_2PO_4	3×10^{-2}	3×10^{-2}	3×10^{-2}
$(NH_4)_2NO_3$	5×10^{-2}	0	0
$(NH_4)_2HPO_4$	0	1.7×10^{-2}	2.0×10^{-3}
Glucose	2.2×10^{-1}	7×10^{-3}	2.8×10^{-2}
pH	6.7	6.7	6.7

* from Cooper et al. (1981)
** varied during medium testing

their effects on subsequent generations. However, the principle of continuous phasing is based on the growth and doubling of the cell and the control must be on this level. All components of the medium will in some way affect growth, for example by modulating the rate of some metabolic process, but these can be defined as rate limiting as opposed to growth limiting. In chemostat operation, where growth is continuous instead of semi-continuous, the distinction between rate limiting and growth limiting is not possible. Growth limitation cannot be observed without being able to identify a stop/start point.

From Table 1 it can be seen that the differences between the original medium from Cooper et al. (1981) and the medium used in these studies are the concentrations of the two divalent cations, manganese and iron, while the carbon and nitrogen were reduced to produce specific limitations. In the medium of Cooper et al. (1981) both carbon and nitrogen are in excess, while there appears to be a deficiency in at least manganese and possibly iron as well. Since it is important to know what is growth limiting, both iron and manganese were initially increased to relatively high concentrations. Then, batch fermentation studies were performed to monitor biomass as the nitrogen levels were varied. The results confirmed that with a concentration of $(NH_4)_2HPO_4$ of 0.004 molar or less, nitrogen was exhausted at the time of maximum biomass concentration and that further decreasing the nitrogen resulted in a directly proportional decrease in maximum biomass concentration. This medium was subsequently used for the nitrogen-limited continuous phasing experiments. It is important to note that although nitrogen is the growth limiting nutrient, the rate limiting effects of the other nutrients can also be studied by either increasing or decreasing their concentrations over a range that still results in absolute exhaustion of the nitrogen. Once the nitrogen is no longer completely used, then the manipulated component has switched to become growth limiting and nitrogen is in excess. This switch-over point is likely to reveal a very different metabolic response from the cell population (Dawson 1972). Although only preliminary phasing experiments were operated under carbon limitation, the same procedure was used to determine the C-limited medium.

During continuous operation of the phasing system, medium was stored in a pair of 10 liter Nalgene bottles connected in parallel to the control solenoid valve on the inlet to the dosing vessel. The medium was replenished periodically as needed by aseptically transferring 8 liters of a glucose solution and 8 liters of a mineral salt solution (which had been autoclaved separately) into the two storage bottles. Generally 15 liters of medium was sufficient for about five days of operation.

CHAPTER 3

APPARATUS

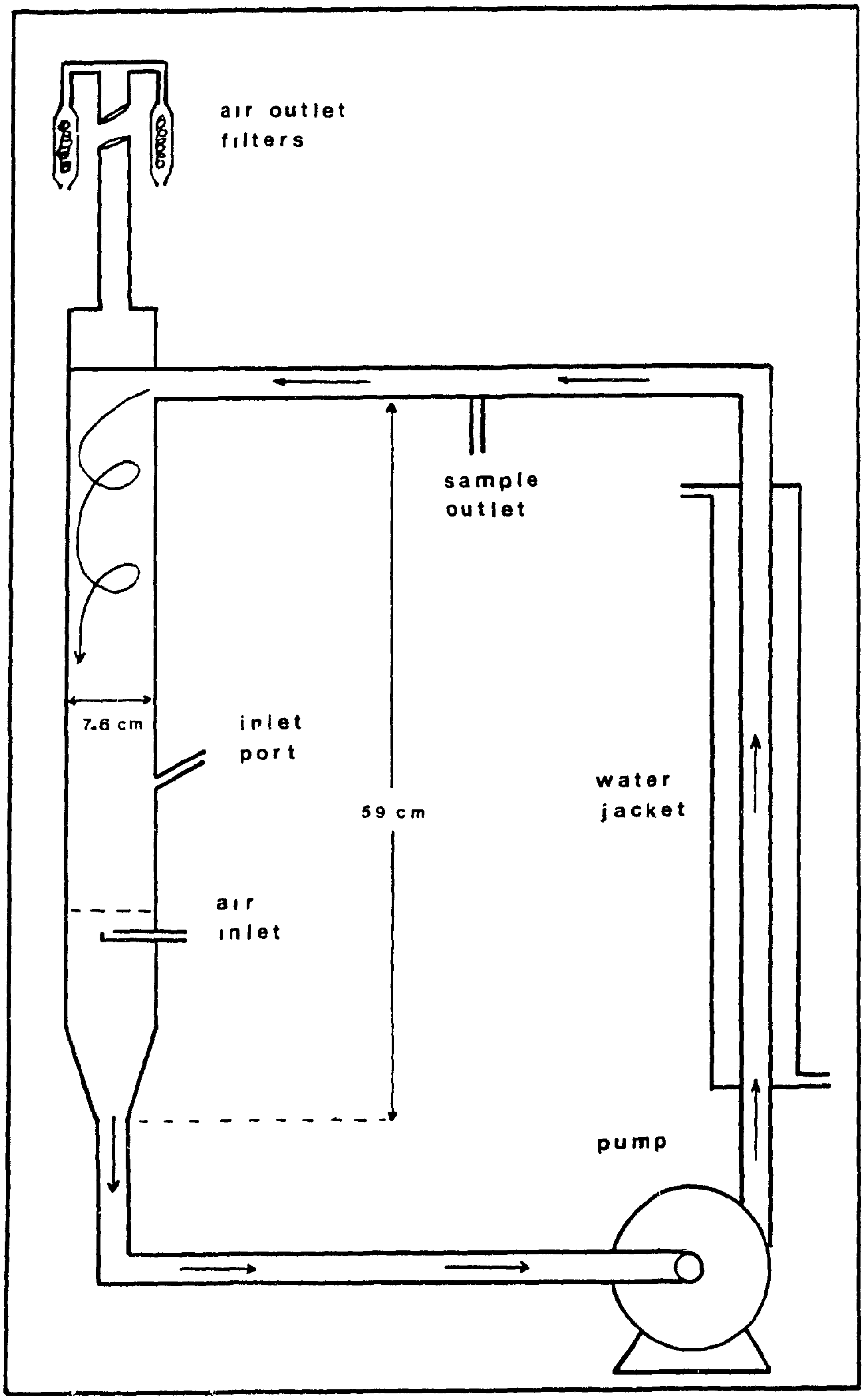
3.1 The Cyclone Column Reactor

All fermentations were performed in a reactor referred to as a cyclone column. The cyclone column was used extensively by Dawson (1963, 1974) and Dawson et al. (1971) for growth of Candida utilis at both the bench and pilot plant scales and is the subject of a Canadian patent, recently licensed to W.H.E. Process Systems Limited. Bench scale units were used for these studies on Bacillus subtilis, one of which is illustrated in Figure 6. The working liquid volume could be varied between about 400 mL and two liters, however, normally 800 mL was used in order to provide adequate head space for the swirling film and foam. Mixing is accomplished by recirculating the contents through a side arm by means of a 0.02 horsepower centrifugal pump (March Model MDX). The contents re-enter the column at the top via a tangential entry resulting in a cyclone action and a swirling falling liquid film on the inside wall of the reactor. Air is introduced into the column about 15 cm from the bottom and rises countercurrent to the liquid flow, escaping out the top after passing through a condenser and glass wool filters. Oxygen transfer from the gas into the liquid phase occurs across the falling film and also from bubbles entrained in the recirculation

Figure 6

The Cyclone Column Reactor

Shown is the basic apparatus used for studying bacterial growth.


air outlet filters
sample outlet
7.6 cm
inlet port
59 cm
water jacket
air inlet
pump

loop.

The cyclone column was selected for these studies because the bench scale units have several advantages when compared to conventional stirred tanks. These advantages include:

1) Uniform mixing with no dead spaces is accomplished with a high recirculation rate (7.9 liters per minute or a 6 second turnover rate with an 800 mL volume) and a design that minimizes any internal surfaces that are not subjected to liquid travelling at high velocity (46 cm per second in the side arm). Unlike a stirred tank, there is no need for internal baffles, coils for temperature control or air sparger rings.
2) There are no aseptic seals; sterilization of the complete unit is accomplished by simply unclipping from the mounting board and placing it in the autoclave. The magnetically-driven recirculation pumps are rated for continuous duty, the heads being sterilizable after addition of aluminum clamping plates. The absence of seals and gaskets in addition to the light weight, all glass construction results in a simple but reliable laboratory reactor.
3) The pumped recirculation loop ensures that uniform samples can be obtained for both in situ analysis with a dissolved oxygen probe and for external laboratory analyses. This is of special importance for operating

continuously or semi-continuously when representative aliquots of the reactor contents must be removed to ensure constant conditions within the reactor.

3.2 Ancillary Control Equipment

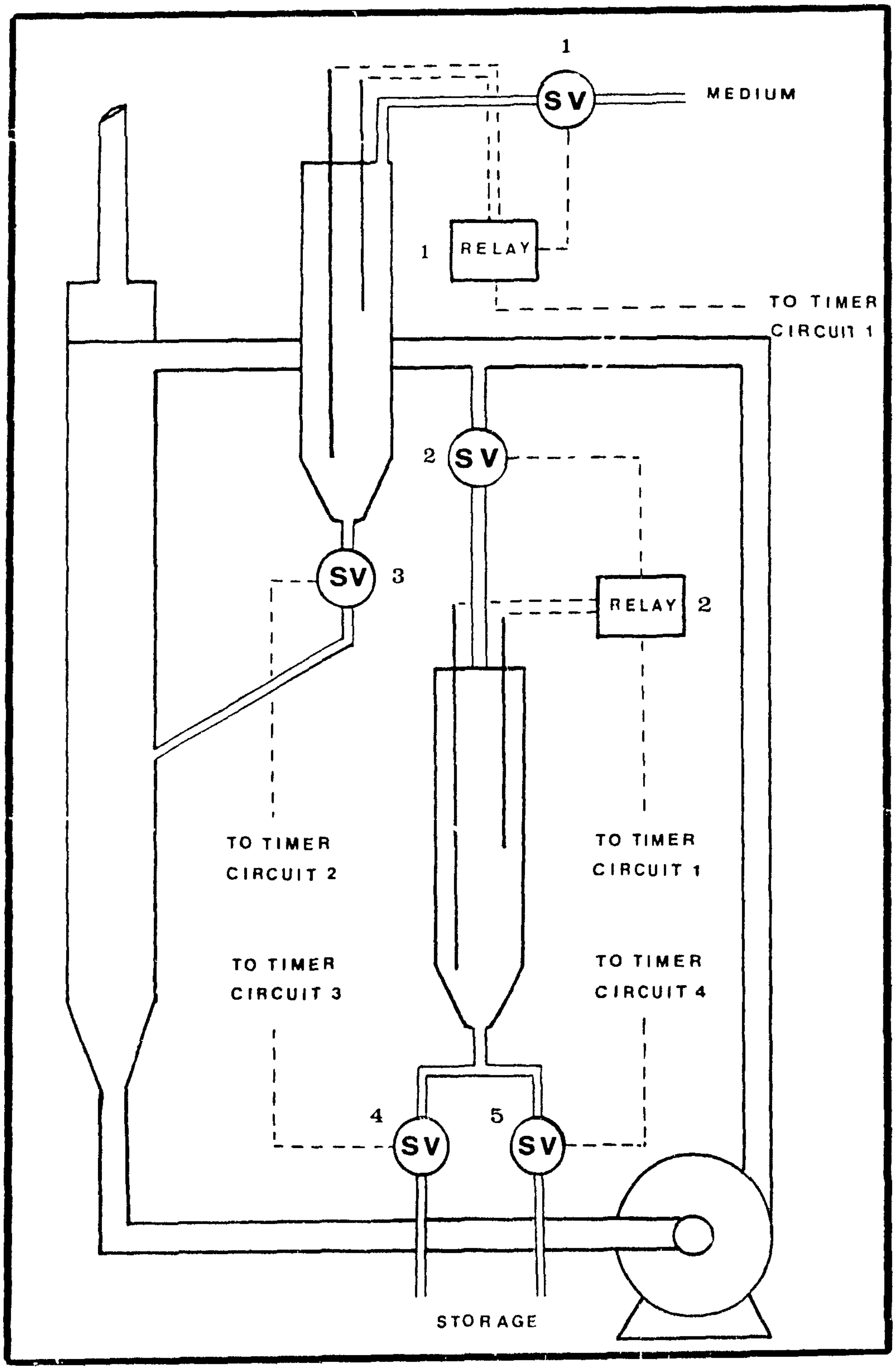
Operation of the cyclone column reactor in a batch mode requires minimal ancillary equipment other than the recirculating water bath (Haake Model FE 2) for temperature control and a rotameter (Brooks Model 1355XB1B1AAA) for control of the air supply. Samples are obtained manually during the batch from a sterile sample tube at the top of the recirculation loop. Periodic addition of the nutrient solution and removal of the cell broth, as occurs during continuous phasing, requires considerably more hardware.

Sterilizable solenoid valves (Skinner Model V52LB2052) were used to control the flow of fresh medium from the medium storage into an intermediate holding vessel (Figure 7) and subsequently into the reactor. The same type of valves controlled the flow of cell broth from the reactor into an intermediate harvesting vessel before emptying it into a refrigerated sample container. The intermediate vessels were used to measure liquid volumes, thus ensuring that the dose of fresh medium and harvested broth were both equal to one half of the total working liquid volume in the reactor. Control over the solenoid valves and, therefore, the dosing

Figure 7

Phasing Control by Timer and Level Probes

Shown are the ancillary control equipment used to provide the periodic addition of fresh nutrients and harvesting of cell broth. (SV = solenoid valve)


1
SV
MEDIUM
1
RELAY
TO TIMER CIRCUIT 1
2
SV
SV
3
RELAY
2
TO TIMER CIRCUIT 2
TO TIMER CIRCUIT 1
TO TIMER CIRCUIT 3
TO TIMER CIRCUIT 4
4
SV
5
SV
STORAGE

and harvesting cycle, was accomplished using two different methods, each requiring different hardware.

The first method used for control of the continuous phasing cycles was developed by Dawson (personal communication 1986). The dosing and harvesting volumes were measured in the intermediate vessels with the use of metal electrodes that formed a conductivity bridge when the liquid level contacted the electrodes. Completion of the circuit closed a relay (Fisher Model 32) which in turn closed the solenoid valve that controlled the flow of the incoming liquid. The dosing or harvesting volumes could be adjusted by manually changing the heights of the electrodes in the vessels. Timing of the dosing and harvesting sequence was accomplished with a timer/relay box (Chontrol Model CD-4) that had four programmable outlets. Each outlet could be opened or closed at any time in a repeated sequence with a period equal to any integer multiple of one hour. Referring to the valve numbers in Figure 7, a typical control sequence operated as follows:

i) Timer circuit (outlet) #1 closes, thereby energizing the relay box #1 which opens the solenoid valve (SV) #1. Fresh medium flows into the intermediate dosing vessel until the liquid level contacts the two electrodes. This completes the circuit and closes SV #1 via the relay box #1, thereby stopping the flow of fresh medium at the desired volume.

ii) The same logic applies to the harvesting sequence (SV #2 and relay #2) which is also controlled by the timer circuit #1. Therefore, both intermediate vessels are filled with the appropriate volumes simultaneously.

iii) After a delay of one or two minutes, timer circuit #2 energizes SV #3, allowing the fresh medium to flow from the intermediate vessel into the reactor. This returns the working liquid volume to its full complement.

iv) After SV #3 is de-energized to prepare for the next complete cycle, either circuit #3 or #4 energizes SV #4 or #5 respectively. This empties the contents of the harvesting vessel into a refrigerated sample container. Operation of SV #4 and #5 alternates with a period equal to twice the total cycle time, thus allowing two complete cycles without operator attendance. The sampling valve is de-energized after a delay of about five minutes to ensure that the harvesting vessel has completely drained.

v) The timer/relay control repeats the entire harvesting and dosing sequence after a time period selected to approximate the cell doubling time.

As illustrated in Figure 7, the harvesting vessel was positioned such that the broth was removed from the top portion of the recirculation loop, thereby ensuring a homogeneous sample. Two problems affected the accuracy of the harvested volume: both the amount of foam in the harvesting vessel and fouling of the electrodes affected their sensitivity. The effects of the foam level on the volume measurement were partially compensated for by reducing the circuit sensitivity by an adjustment on the relay box. Also, lengthening the harvesting time allowed the foam to collapse. These strategies were, however, only partial solutions and some variation in harvesting volume and, therefore, reactor volume was encountered. Manual adjustment of the electrode height was resorted to in periods of rapid change but this was considered to be undesirable because of the continuous operator attention that was required. In spite of these problems a run of 100 consecutive generations of cells was successfully completed using this method for control.

Use of the four-circuit timer/relay also limited the control flexibility; only four circuits could be controlled and the cycle time (time after which the logic sequence was repeated) could only be set in integer multiples of one hour. This allowed for only two sampling valves in addition to the dosing and harvesting valves. The limitation on the cycle timing was initially not considered to be of great importance. Based on the experience of Dawson (1971, 1985) with yeast, the cells were expected to have sufficient metabolic flexibility to compensate for "non-ideal" cycle times.

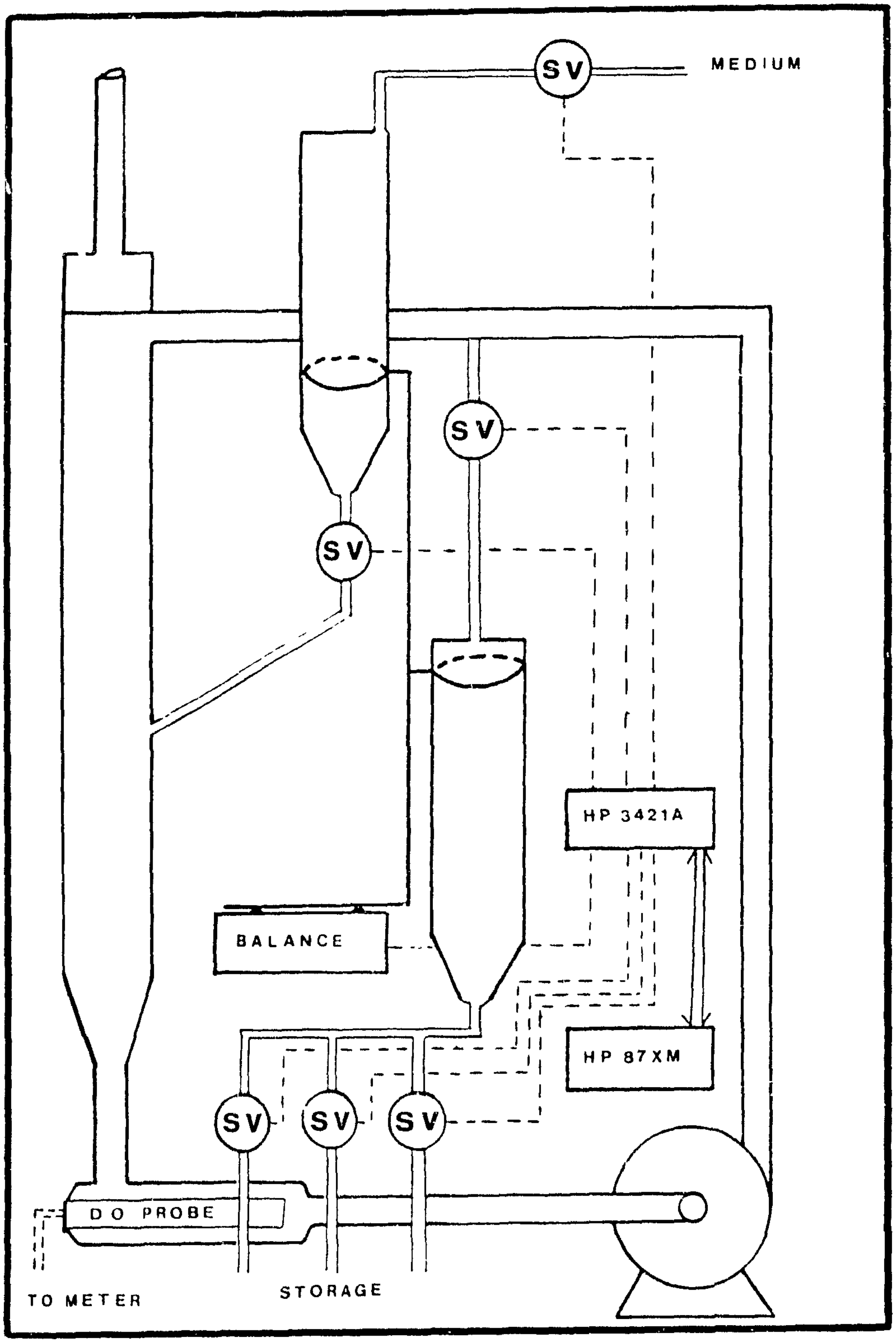
Both the control flexibility and accuracy were improved with the use of a computer in conjunction with a data acquisition and control interface device connected to an electronic balance. The computer was a Hewlett Packard Model 87XM, connected via an HP-IB parallel interface bus to a Hewlett Packard 3421A data acquisition and control unit which performed the A/D conversion of the signal from the balance (Precisa Model 6000 D). Peripheral computer equipment consisted of a dual disc drive (HP Model 9121) and a printer HP Model 82906A) all connected via the HP-IB parallel interface. The set-up of the system is illustrated in Figure 8 which shows the HP 3421A acting as a bi-directional interface. The HP 3421A is capable of being connected to up to 30 devices through three I/O cards. Each card can be configured to receive 8, 9 or 10 analogue inputs with up to two channels functioning as control relays. In this system maximum use was made of the control relays, connecting to six solenoid valves. Analogue inputs consisted of 0-1.0 volt signal from the electronic balance (Precisa Model 6000D) proportional to a 0-6000 gram weight and, later, a 0-0.8 volt input from the Pegasus dissolved oxygen meter.

Both the dosing and harvesting vessels were mounted on a stand which was placed on the electronic balance. Liquid flowed in and out of the vessels through flexible latex tubing. The computer was able to monitor the volumes in either vessel by means of the digital signal from the HP3421A which had converted the analogue output from the balance. The HP87XM computer has several internal timers that are accurate to within a millisecond over 24 hours, therefore virtually any

Figure 8

Phasing Control by Computer and Electronic Balance

Shown are the ancillary control equipment used to provide the periodic addition of nutrients and harvesting of cell broth based on computer programmes. (SV = solenoid valve)


SV
MEDIUM
SV
SV
HP 3421A
BALANCE
HP 87XM
SV
SV
SV
DO PROBE
TO METER
STORAGE

cycle time could be selected and any dosing or harvesting volume accurately obtained, independent of foaming. In addition, three sample valves could now be connected, extending the period of automatic operation before sample containers were replaced.

The control programmes (written in HP Advanced Basic) went through a series of modifications designed to increase both the accuracy and flexibility of the system. The programmes are described in Sections 4.2.1 and 4.3.1. It was, however, clearly evident that the addition of the computer control significantly increased the reliability and potential scope for continuous phasing of microbial cultures.

CHAPTER 4

EXPERIMENTATION

4.1 Batch Culturing

4.1.1 Procedure

The main purpose of growing batch cultures of *B. subtilis* was to determine the maximum specific growth rate that could be attained in a particular nutrient medium. This occurs during the logarithmic phase which is considered to be the only reproducible phase of a batch culture (Dawson 1985a). The maximum specific growth rate obtained from the batch data gives a starting point for selection of an appropriate period for the nutrient cycles imposed on the cells during continuous phasing.

Two types of batch cultures were grown: the conventional closed batch and a modification to the technique referred to as consecutive batches. In the conventional batch technique the inoculum was prepared by first inoculating a shake flask from a nutrient agar plate. When significant growth had been obtained in the flask (after a variable length of time), a volume of broth equal to 1% of the reactor volume was used as the inoculum. Care was taken to duplicate the procedure as closely as possible for each batch, however, it was not possible for each inoculum to be identical with respect to the metabolic state of the cells. The technique of consecutive batches was developed to overcome this problem.

Consecutive batches differ from ordinary batch culturing of cells in that the inoculum is obtained from a preceding batch that was operated in a manner identical to the batch preceding it. This technique can be continued indefinitely and in this way is consistent with the operational strategy employed with continuous phasing. The nutrient environment is varied in a periodic manner that is repeated over many generations of cells, thereby revealing a whole range of potential effects that become independent of a single inoculum (as in a conventional single batch). The results can be interpreted from an historical perspective that spans several nutrient cycles and many generations of cells.

The basic difference between consecutive batches and continuous phasing is the number of generations of cells that grow during each nutrient cycle. By definition, continuous phasing permits one generation to double while consecutive batches can span from two to at least ten generations of cells per cycle. Unlike conventional batches, the quantity of inoculum should be selected so as to permit an integer number of generations to be produced during a single batch cycle. Otherwise interpretation of the results becomes more difficult. The procedure required that two cyclone column reactors be operated alternately. In this way the delay between the completion of one batch and the beginning of the next was limited to less than one minute.

4.1.2 Results

Figure 9 illustrates the change in surface tension and optical density of the culture during the batch growth of B. subtilis in the original medium of Cooper et al. (1981) with 20 g/L of glucose. Except for an anomaly at ten hours, the surface tension remained between 56 and 60 mN/m until between 11 and 12 hours. The most rapid decrease in surface tension began at the point the biomass concentration was about one half of the maximum (1.3 absorbance units). Each absorbance unit corresponded to approximately 0.38 grams of dry cells per liter and dilutions were performed if the absorbance exceeded 1.0. By 14 hours the surface tension had dropped to less than 29 mN/m and growth stopped one hour later, reaching a maximum biomass concentration of about 1.0 g/L. The minimum biomass doubling time during the logarithmic growth phase was about 1.2 hours.

A similar pattern was obtained with batch growth on the nitrogen limited medium illustrated in Figure 10. Although, in the batch illustrated in Figure 10 there was a more extended lag period and the minimum biomass doubling time was about 1.8 hours. Again the surface tension began the most rapid decline at the time at which about one half of the maximum biomass concentration had been achieved, in this case

Figure 9

A Batch Culture of *B. subtilis* in the Original Medium of Cooper et al. (1981)

Shown is the change in surface tension (△) and biomass concentration, as monitored by optical density at 600 nm (●).

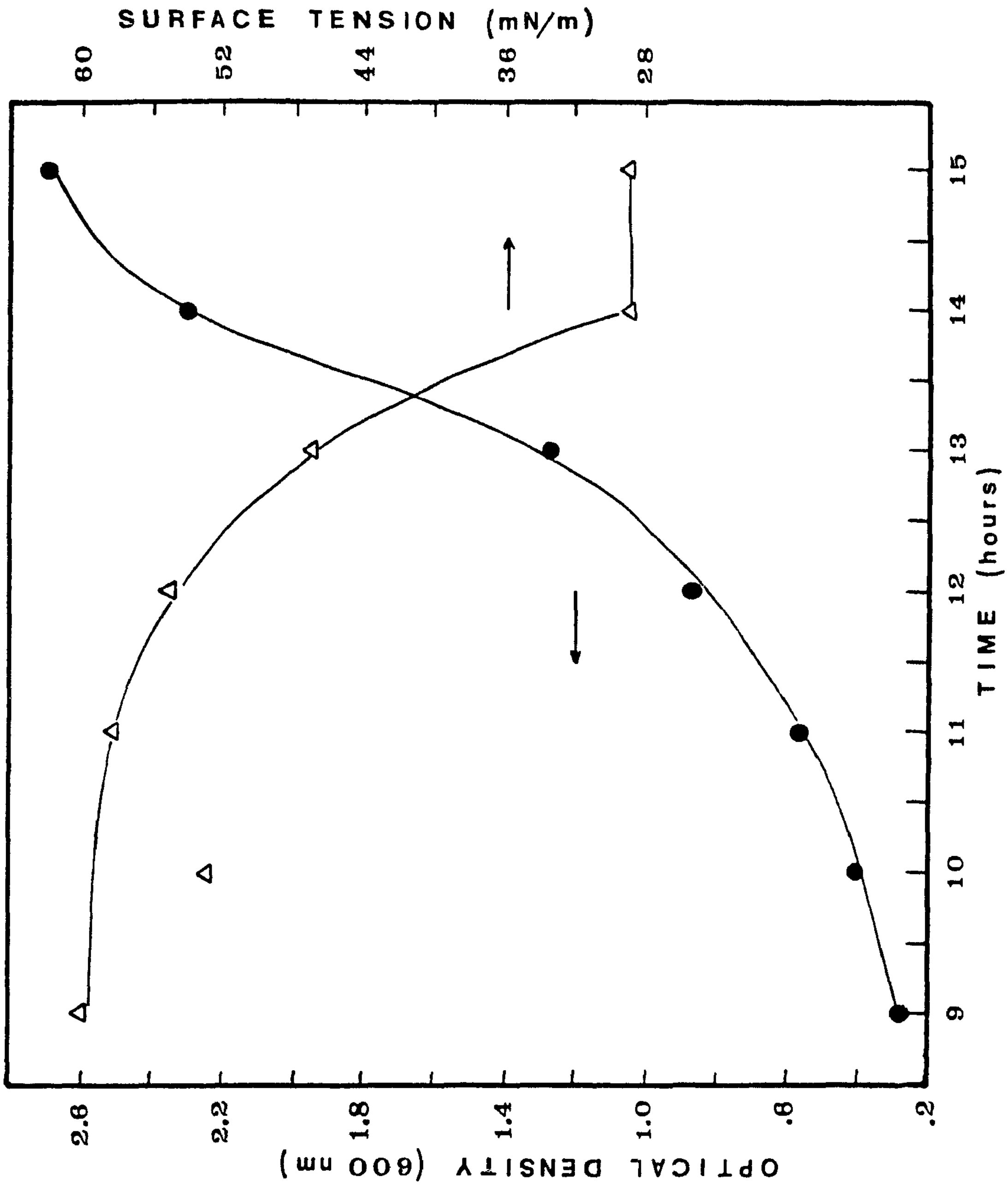
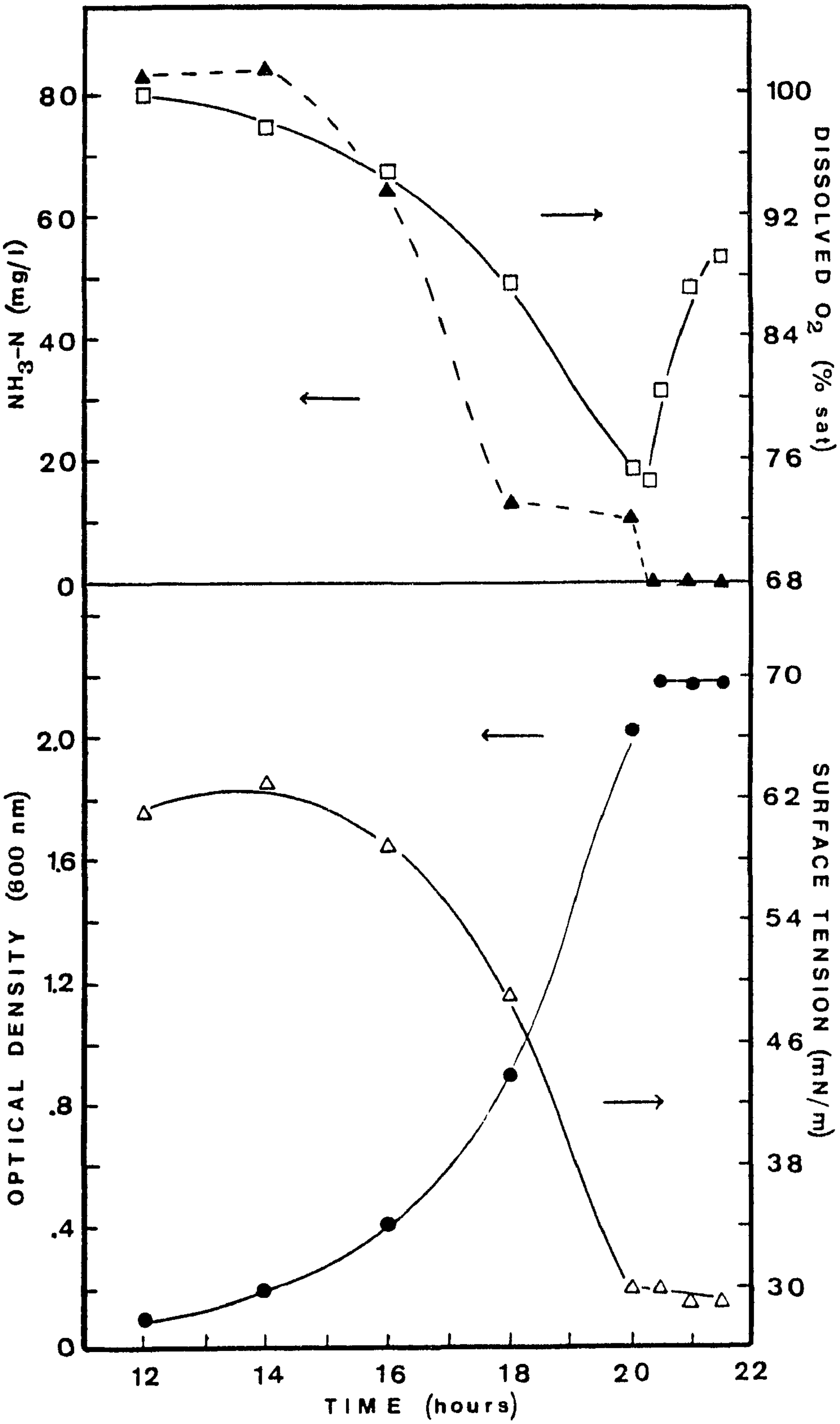

SURFACE TENSION (mN/m)
60
52
44
36
28
OPTICAL DENSITY (600 nm)
2.6
2.2
1.8
1.4
1.0
.6
.2
TIME (hours)
9
10
11
12
13
14
15

Figure 10

A Batch Culture of B. subtilis in a Nitrogen-limited Medium Showing the Minimum Dissolved Oxygen

The batch culture was grown in a medium limited by 4 x 10^{-3} molar diammonium phosphate. The maximum biomass concentration (●) corresponds to minima in the surface tension (△), nitrogen (▲) and dissolved oxygen concentration (□).


NH$_3$-N (mg/l)
80
60
40
20
0
DISSOLVED O$_2$ (% sat)
100
92
84
76
68
OPTICAL DENSITY (600 nm)
2.0
1.6
1.2
.8
.4
0
SURFACE TENSION (mN/m)
70
62
54
46
38
30
12
14
16
18
20
22
TIME (hours)

after about 18 hours. Figure 10 also illustrates the close correspondence between the maximum biomass concentration and minima in the surface tension, nitrogen and dissolved oxygen concentrations.

Figure 11 illustrates batch growth with an initial glucose concentration of 2.5 g/L. A maximum biomass concentration of about 1.1 g/L was achieved, or almost exactly twice what was achieved with 1.25 g/L of glucose. These values correspond to a biomass yield of approximately 43% based on the weight of glucose, or, 73 grams of cells per mole of glucose. Since the yield of biomass based on glucose corresponds very closely in the two experiments, it can be safely assumed that the medium was carbon limited in both cases. The maximum yield for yeast is about 100 grams of cells per mole of glucose (Bailey and Ollis 1977). Figure 11 also shows the very close correspondence between the biomass concentration and the dissolved oxygen concentration. At 24.5 hours the minimum level of dissolved oxygen (about 77% of saturation) was observed, followed by a rapid increase as the growth of the cells stopped. This same phenomenon is clearly seen from Figure 10 where growth was under nitrogen limitation (4 x 10^{-3} molar $(NH_4)_2HPO_4$).

Figure 12 illustrates batch growth prior to beginning continuous phasing by periodic addition of a nitrogen limited medium. Phasing was begun at the 23 hour point, before the maximum biomass concentration was achieved. The surface

Figure 11

A Batch Culture of B. subtilis in Two Carbon-limited Media

Shown are the changes in biomass concentration, as monitored by optical density at 600 nm, for growth in initial glucose concentrations of 2.5 g/L (○) and 1.25 g/L (●). The change in dissolved oxygen concentration (□) is shown for growth in 2.5 g/L glucose.

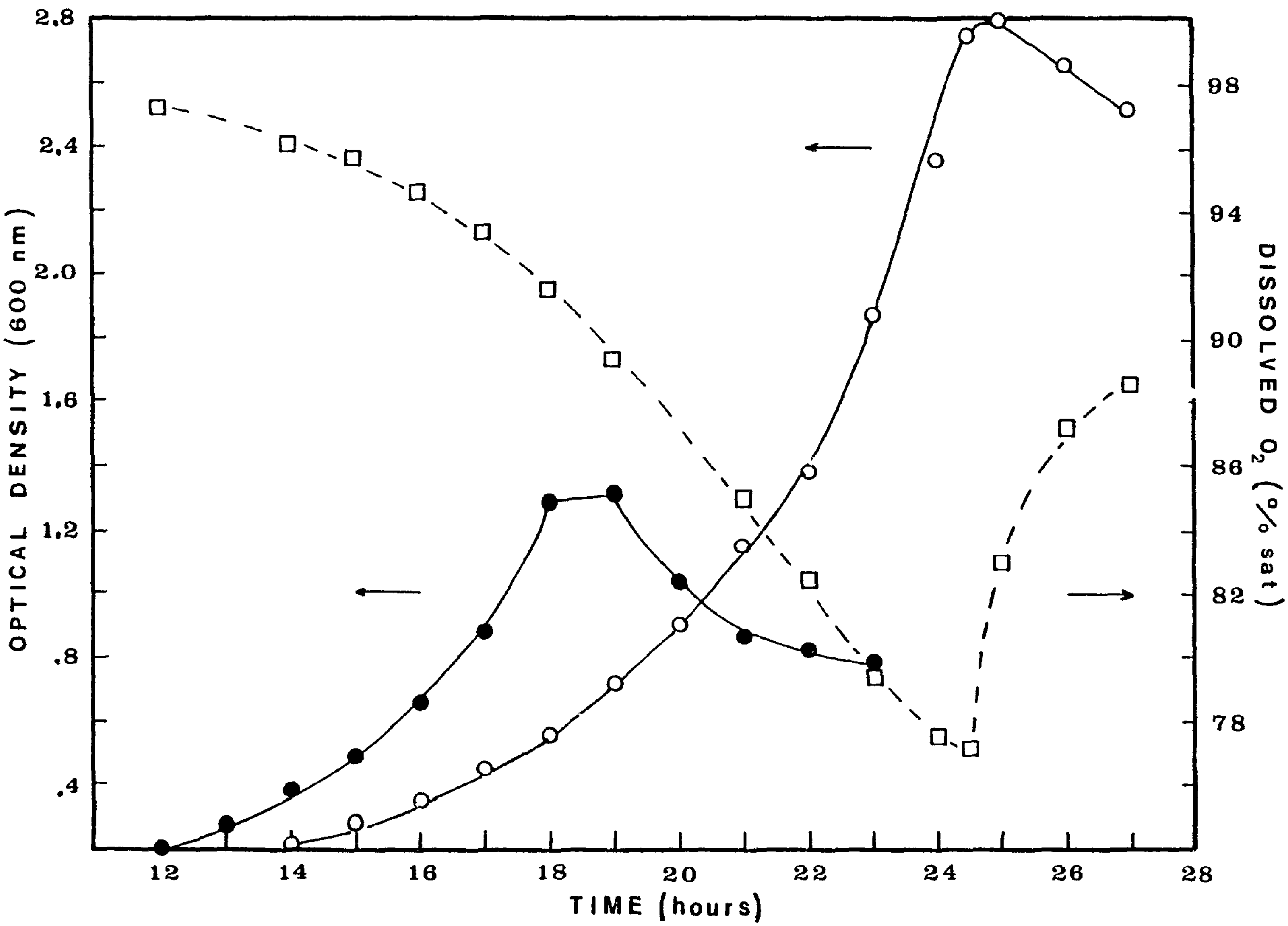
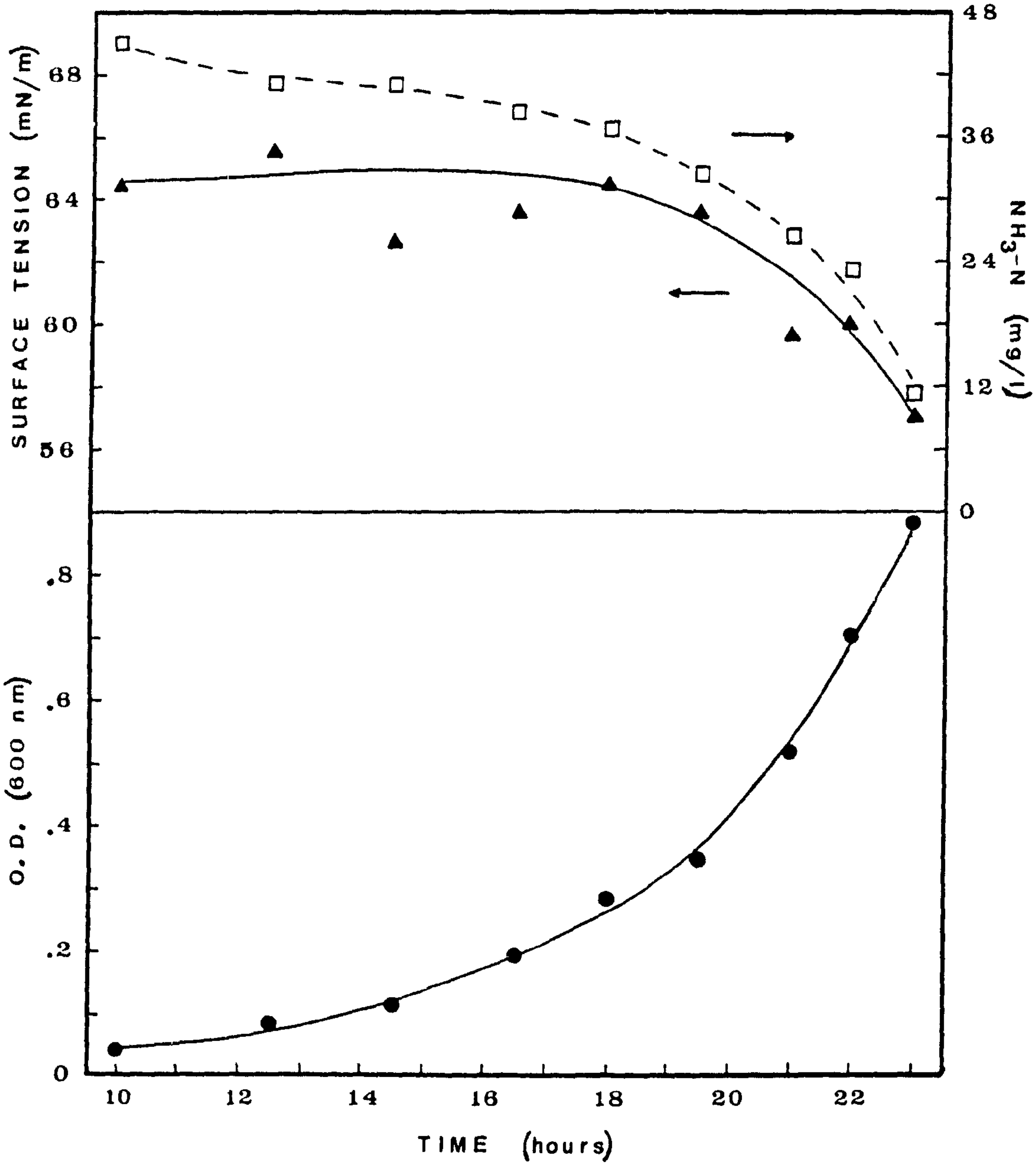

2.8
2.4
2.0
1.6
1.2
.8
.4
OPTICAL DENSITY (600 nm)
98
94
90
86
82
78
DISSOLVED O₂ (% sat)
12
14
16
18
20
22
24
26
28
TIME (hours)

Figure 12

A Batch Culture of B. subtilis Prior to Beginning Continuous Phasing

Shown is the change in surface tension (▲), nitrogen (□) and biomass concentration as monitored by optical density at 600 nm (●). Continuous phasing was begun at 23 hours, prior to achieving the maximum biomass concentration.


SURFACE TENSION (mN/m)
68
64
60
56
NH_3-N (mg/l)
48
36
24
12
0
O.D. (600 nm)
.8
.6
.4
.2
0
10
12
14
16
18
20
22
TIME (hours)

tension had just started to rapidly decrease as the rate of nitrogen utilization was rapidly increasing and the cells were in the logarithmic growth phase. The minimum biomass doubling time was about 2.6 hours. A summary of the minimum biomass doubling times achieved during batch growth in a variety of media is presented in Table 2.

Three different consecutive batch "runs" were performed, each run consisting of at least four batch cultures under nitrogen limitation. The first consecutive batch is illustrated in Figures 13 and 14, showing the variation in growth and nitrogen utilization. The initial batch was inoculated from a shake flask (1% by volume of the reactor) while each subsequent batch was inoculated with 1% by volume from the preceding batch, after 20 hours of growth in each case. Three different patterns of growth resulted. The first batch was associated with a very long lag phase, about 16 hours, followed by very rapid growth and uptake of nitrogen over the next four hours. This growth continued into the next batch which showed no lag, consuming all the nitrogen by 16 hours. Batches three and four revealed growth patterns that were essentially identical, although there was a slight difference in the rate of nitrogen uptake. This last growth pattern differs significantly from the classical batch growth "cycle". There is no lag phase but, instead, there appears an intermediate "stationary" phase, between four and eight hours, with logarithmic growth continuing until about 16

Table 2

Biomass Doubling Times During Batch Growth

Medium	Limiting nutrient	Mn conc. in medium (moles/L)	Min. Td (hours)
Cooper's*	?	0	1.2
C-ltd.	glucose at 0.014 molar	5×10^{-5}	2.9
C-ltd.	glucose at 0.007 molar	3×10^{-4}	2.3
N-ltd.	$(NH_4)_2HPO_4$ at 0.002 molar	3×10^{-4}	2.6
N-ltd.	$(NH_4)_2HPO_4$ at 0.004 molar	3×10^{-4}	1.7

* from Cooper et al. (1981)

Figure 13

Variation in Growth During Consecutive Batches with Nitrogen-limitation

Biomass concentration, as monitored by optical density at 600 nm, is shown for four consecutive batches grown in a medium limited by 2 x 10^{-3} molar diammonium phosphate. Batch 1 (○) was inoculated from a shake flash, batch 2 (●) from batch 1, batch 3 (△) from batch 2 and batch 4 (▲) from batch 3.

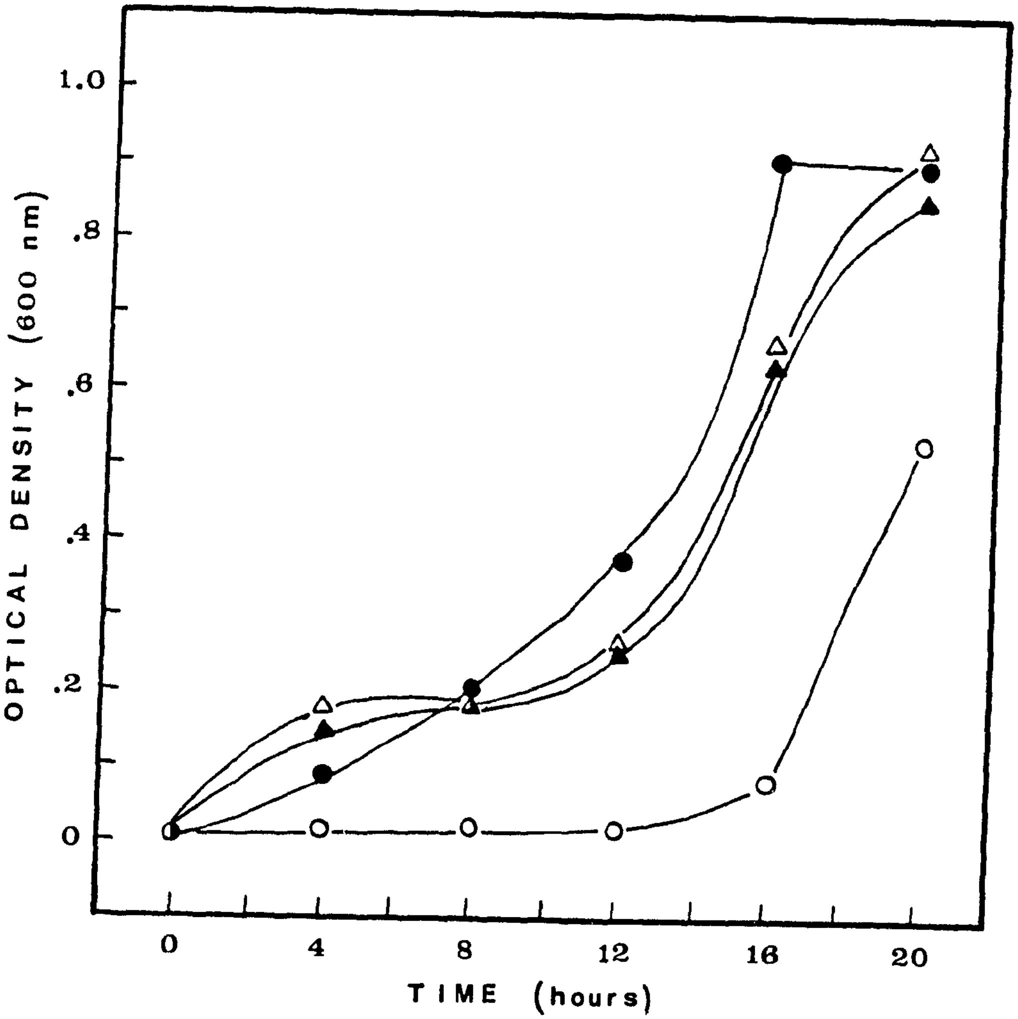
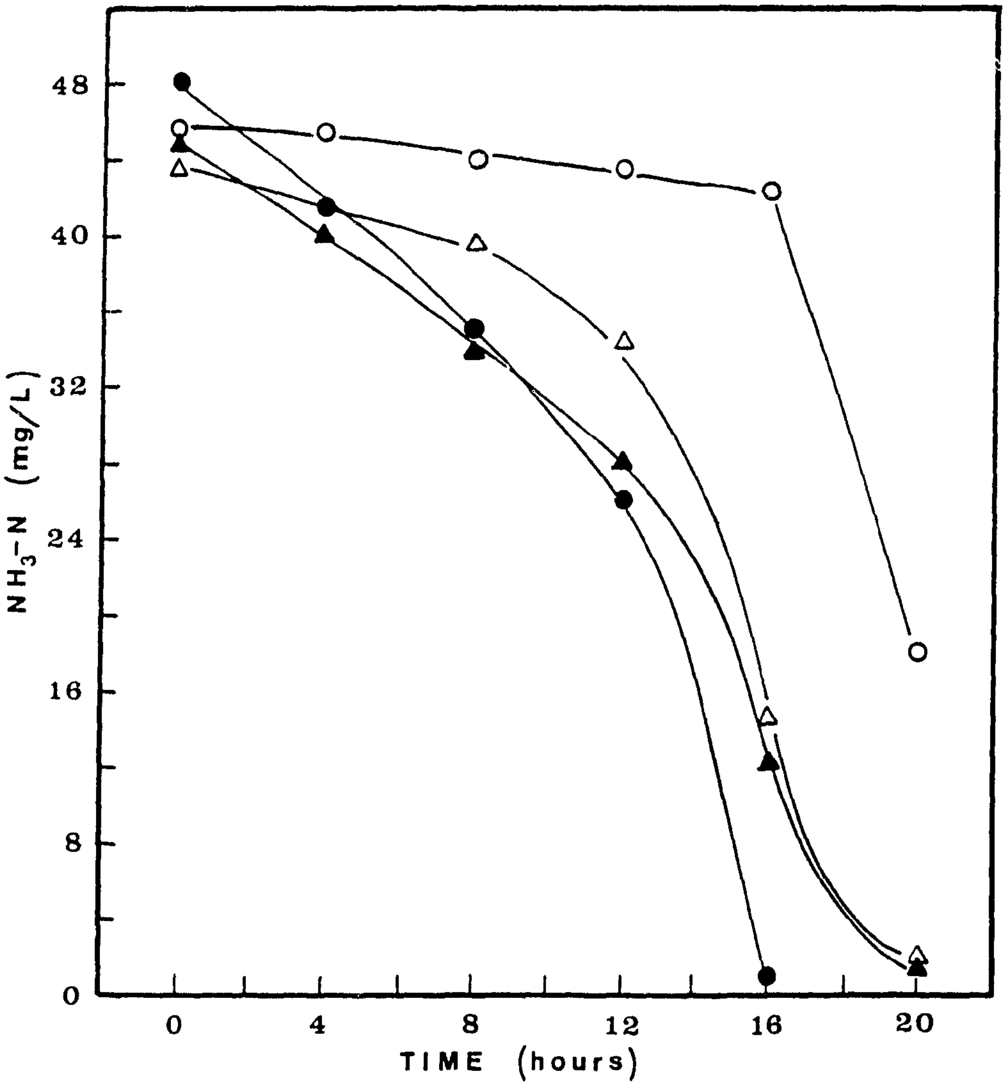

1.0
.8
.6
.4
.2
0
OPTICAL DENSITY (600 nm)
0
4
8
12
16
20
TIME (hours)

Figure 14

Variation in Nitrogen Utilization During Consecutive Batches of *B. subtilis*.

The change in ammonia nitrogen concentration is shown for four consecutive batch cultures. Batch 1 (○) was inoculated from a shake flash, batch 2 (●) from batch 1, batch 3 (△) from batch 2 and batch 4 (▲) from batch 3.


48
40
32
24
16
8
0
NH_3-N (mg/L)
0
4
8
12
16
20
TIME (hours)

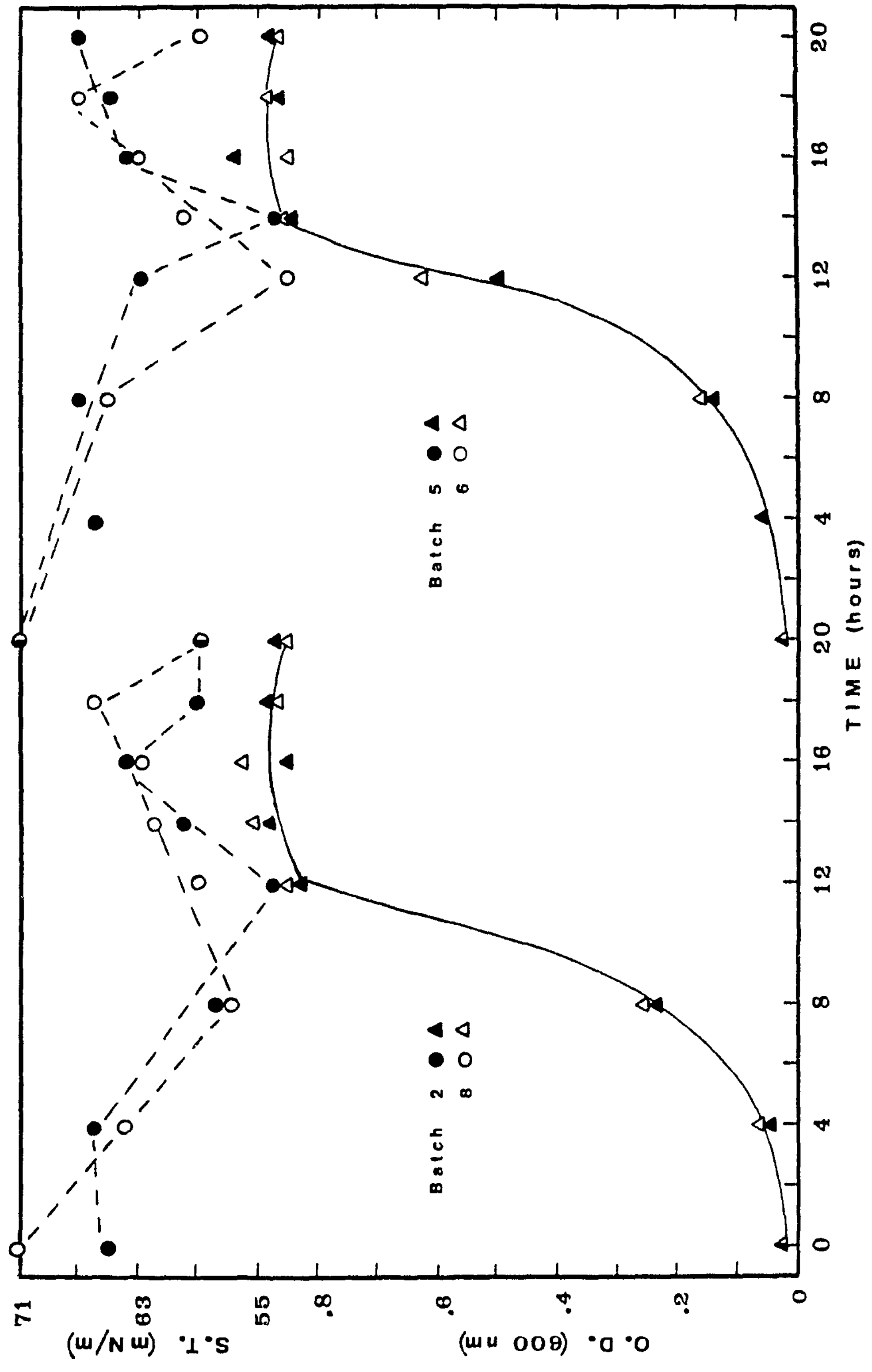
hours. The growth rate then appears to slow down, with the limiting nitrogen reduced to between 1.5 and 2.0 mg/L by 20 hours. Batches two, three and four all achieved minimum biomass doubling times of about 3.0 hours.

For the second set of consecutive batches, the volume of inoculum was increased to 1.6% of the working liquid volume while maintaining the 20-hour period per batch. This would permit a maximum of six biomass doublings, corresponding to a maximum average doubling time per generation of 3.3 hours. This calculation assumes consistent doubling times for each generation but does not require that the cells be synchronized. The experiment was continued for 8 batches or 48 generations. Figure 15 illustrates the results from batches two, five, six and eight. The average doubling times were considerably faster than 3.3 hours, averaging between 2 and 2.3 hours. Two patterns of growth emerged, with cycles two and eight being completed about two hours earlier than the other cycles as a result of a reduced lag phase. However, the most interesting effect is revealed by the surface tension data. Unlike the conventional batches where the minimum surface tension occurred at the end of growth and then remained low, the consecutive batches show a changing pattern where the minimum occurred at any time between 8 and 14 hours, after which there was a significant increase and in 3 of the batches another final decrease. Therefore, what appeared in the conventional batch culture technique as a simple growth

Figure 15

Consecutive 20-hour Batch Culture Using a 1.6% Inoculum

Shown are the changes in surface tension, (○) and (●), and biomass concentration, (△) and (▲), for consecutive batches 2, 5, 6 and 8.


71
63
55
S.T. (mN/m)
.8
.6
.4
.2
0
O.D. (600 nm)
0
4
8
12
16
20
4
8
12
16
20
TIME (hours)
Batch 2 ● ▲
8 ○ △
Batch 5 ● ▲
6 ○ △

associated phenomenon has been revealed as having a separate pattern that does not necessarily correlate with growth.

This same phenomenon is revealed by the 12-hour consecutive batches (Figure 16) with a 12.5% inoculum and 3 cell generations per batch. In batches one, two and four the population behaves as in the conventional batch with surface tension reaching a minimum at the end of growth. However, in cycles three and five the minimum is reached after two and four hours respectively, followed by a rapid increase and then no change until the end. The average specific growth rate is less than in the 20-hour consecutive batches with typical biomass doubling times of 3.3 hours, although the fifth batch grew significantly faster with a minimum doubling time of 2.7 hours. The rate of nitrogen uptake became more uniform for each batch as the experiment progressed.

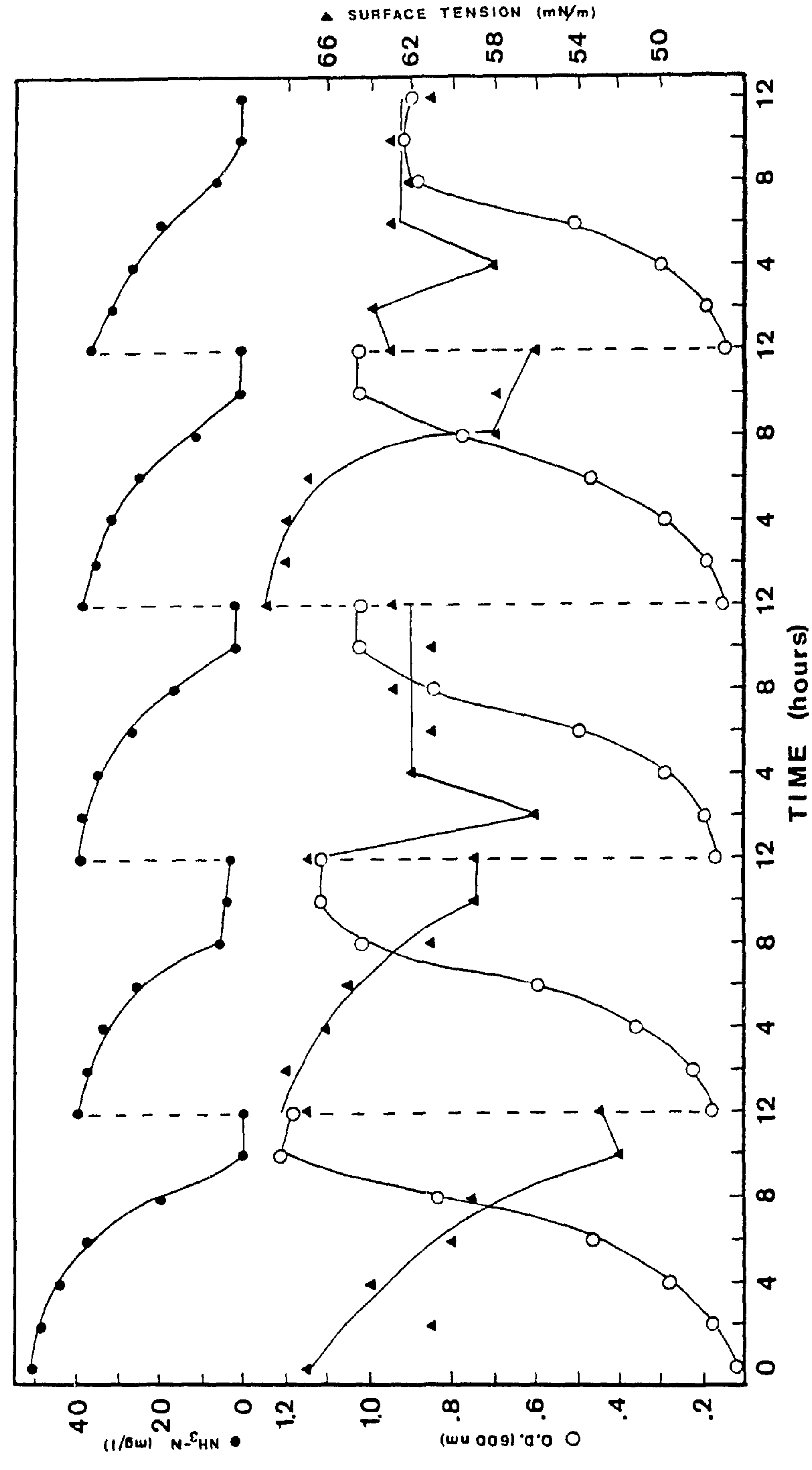
4.1.3 Discussion

The growth of B. subtilis in conventional batch culture suggests that most of the surfactant production occurs in the latter half of the logarithmic growth phase. This is consistent with the experience of Vater (1986), even though the batch growth "cycle" is mistakenly considered to be synonymous with the cell cycle. Since the batch culture spans several cell generations, there is no reason to expect that cell cycle events can be revealed.

Figure 16

Consecutive 12-hour Batch Culture Using a 12.5% Inoculum

Shown are the changes in nitrogen concentration (●), biomass concentration as monitored by optical density at 600 nm (○) and surface tension (▲) for five consecutive batch cultures limited by 2 x 10^{-3} molar diammonium phosphate.


▲ SURFACE TENSION (mN/m)
66
62
58
54
50
● NH_3-N (mg/l)
40
20
0
○ O.D. (600 nm)
1.2
1.0
.8
.6
.4
.2
TIME (hours)
0
4
8
12
4
8
12
4
8
12
4
8
12
4
8
12

Although the duration of the lag phase was highly variable, the conventional batch cultures provided consistent performance during the exponential or logarithmic growth phases. Therefore, quantitative information was obtained that related the composition of the nutrient medium to the maximum specific growth rate. Based on a rather limited number of experiments it seems that at least nitrogen and manganese affect the specific growth rate significantly. For example, doubling the concentration of $(NH_4)_2HPO_4$ in the medium to 4 x 10^{-3} molar, reduced the minimum biomass doubling time from 2.9 to 1.7 hours. Based on this information for carbon or nitrogen limited media, it was possible to estimate a period of nutrient addition that was likely to provide stable operation for continuous phasing. Stable operation is meant to imply constant metabolic performance from generation to generation, as monitored by measurements such as biomass concentration and surface tension. Some indication that this stability would perhaps be difficult to attain was revealed by the new technique of consecutive batch cultures.

At least some of the variability in the lag phase of growth should be eliminated by providing an inoculum in the same metabolic state for the start of each batch. Eventually, this should occur while running consecutive batches, assuming that a consistent growth pattern develops. It is less certain whether the technique would reveal different phenomena during other phases of growth, for example, the logarithmic phase.

The results from the three consecutive batch experiments indicate that more than the lag phase could be affected. The 20-hour batches with 1.6% inoculum achieved minimum biomass doubling times of 2 to 2.3 hours compared to 2.9 hours with 20-hour batches using a 1% inoculum or conventional batches. This is a 20 to 30% increase in "maximum" specific growth rate without changing the composition of the nutrient medium. An opposite effect, however, was achieved with the 12-hour batches using a 12.5% inoculum. The biomass doubling times were generally about 15% longer, increasing to 3.3 hours. Based on these results, it seems probable that not only is the metabolic state of the inoculum an important factor, but also the quantity of inoculum. The quantity will affect the number of cell generations that can grow and divide based on the amount of nutrients supplied. It is possible that the technique of consecutive batches could reveal an optimum inoculum size that corresponds to the ability of successive generations to form a hypercyclic organization (Eigen and Schuster 1977, 1978). The conditions for forming a hypercycle are appropriate, since the technique of linking successive batches with the inoculum transforms batch culture from a linear to cyclic process. Furthermore, the batch cycle is composed of interacting component cycles on several levels, including both metabolic and cellular.

In addition to the effects on specific growth rate, the consecutive batches also revealed at least two patterns of surfactant production that appear to be independent of the overall batch growth cycle. This is most clearly seen in Figure 16, illustrating the five consecutive 12-hour batches. Three of the five batches confirm the familiar pattern of surfactant production during middle to late logarithmic phase. However, two batches produced an early rapid decline in surface tension, followed by an increase and then no change throughout most of the logarithmic phase. The technique of consecutive batches, therefore, would indicate that cell growth and other metabolic phenomena should always be interpreted from an historical context. The metabolic/environmental history of preceding generations apparently affects the responses of present and future generations. From these experiments it could not be determined how extensive these effects are, from either a temporal or a spatial perspective. It is expected that under a repetitive environmental stimulus the effects would diminish from cycle to cycle. However, this is just conjecture. Continuous phasing, in which the nutrient cycle corresponds to the cell division cycle, is a more convenient and, potentially, more revealing technique for further investigating this phenomenon. By decreasing the period of the nutrient cycle so that it corresponds to a single cell generation, an entire level of complexity is eliminated. Any metabolic "carry-over" from one generation to the next should be revealed directly.

4.2 Continuous Phasing with Imposed Cycle Times

4.2.1 Procedure

Operation of the reactor in a mode referred to as continuous phasing was considerably more complex than batch culturing and underwent an evolutionary development during the course of the project. As described in Section 1.4 the concept of continuous phasing was utilized extensively by Dawson and co-workers (1965, 1968, 1969, 1972) mainly using *Candida utilis.* The technique requires the periodic removal of one half of the broth followed by replenishment with an equal volume of fresh nutrient. Therefore, for a stable situation to be maintained based on biomass, the period of nutrient addition or "cycle time" must be sufficiently long to ensure doubling of the viable biomass. As a corollary, if samples obtained at the end of successive cycles are consistent with respect to biomass (as monitored by cell number, dry weight or optical density), then doubling of the biomass *must* be occurring during the period of nutrient addition. Furthermore, if growth continues to the end of the nutrient cycle, the cycle time will be equivalent to the cell doubling time. If cell doubling does not occur during the period of nutrient cycling then unstable performance will result, as indicated by changes in the biomass concentration over successive cycles. The stability of the biomass can be monitored with either cell number, dry weight or optical density. Although cell number would be the best measurement, practically it is the most difficult to obtain accurately. Numerous factors can influence the viable cell count, including chains or clumps of cells, shock and cell lysis that occurs during the plating procedure (Postgate 1967). When only small samples of broth could be obtained, such as during second stage studies illustrated in Figures 21 to 23, optical density measurements were used to monitor the biomass concentration. When larger samples could be obtained, such as from the broth harvested at the end of a nutrient cycle, dry weight was generally the preferred measurement.

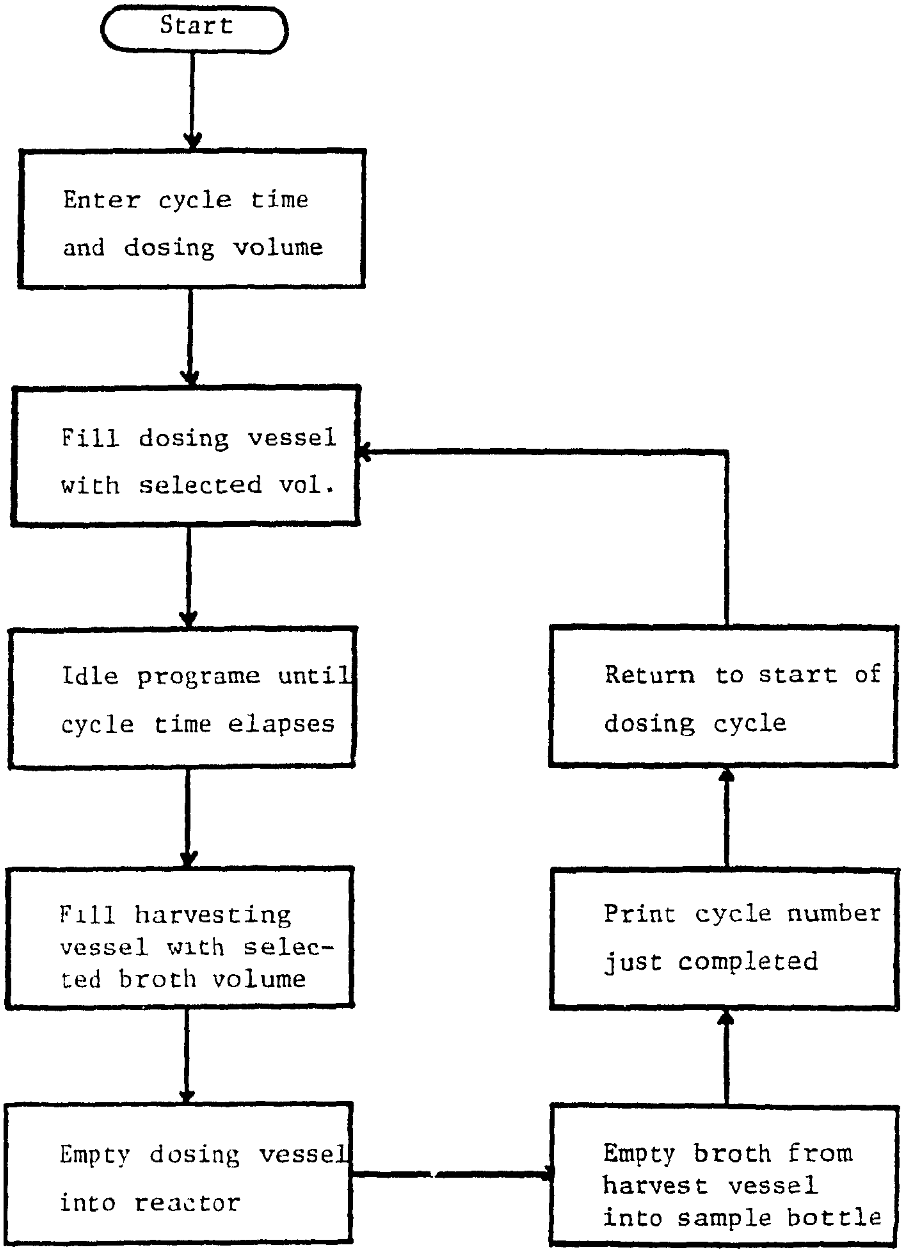
In the past, the control over the periodic harvesting of the broth and dosing with fresh nutrients was accomplished with the use of level probes and solenoid valves, connected to a programmable timer (Dawson personal communication 1986). This method has been described in detail in Section 3.2 and was used for preliminary experiments with carbon limitation and one experiment under nitrogen limitation. However, as a result of copious foam production and lack of control flexibility, this method was updated to include more sophisticated computer control. The apparatus required to implement these modifications has also been described in Section 3.2. In addition, the computer required original software to accomplish the desired control strategy. These programmes underwent development as experience with the system increased and the range of possible control strategies was investigated. The simplest programme essentially replaced the role of the timer in imposing a particular period of nutrient cycling, as well as measuring the appropriate dosing and harvesting volumes via the electronic balance. A simple flow chart in Figure 17 illustrates the logic of Version 1 of the control programme. Complete listings, in HP Advanced Basic, of all the versions are included in Appendix C. Version 2 differs only slightly from Version 1 by including a feature to automatically correct for errors in the measured volumes, achieving approximately 1% accuracy (400 plus or minus 4 ml) each cycle. It was, however, important to try to prevent cumulative errors because the actual working liquid volume in the reactor was not monitored by the computer, only the dosing and harvesting volumes.

The conventional method for continuous phasing requires the selection of a period for cycling the growth limiting nutrient (cycle time). Since the metabolic response of the population will be affected by the cycle time chosen, this response must be interpreted partly in terms of the cycle time. To some extent the selection of the cycle time is arbitrary within the limitations that too short a cycle time will result in washout of the cells and too long a cycle time may result in excessive loss of viability. However, between these two extremes considerable variation is possible depending on the microorganism and the nutrients supplied in

Figure 17

Flow Chart for Control of Continuous Phasing Using Imposed Cycle Times

Shown is a simplified flow chart which forms the basis for computer programmes Versions 1 and 2.


Start
Enter cycle time and dosing volume
Fill dosing vessel with selected vol.
Idle programe until cycle time elapses
Fill harvesting vessel with selected broth volume
Empty dosing vessel into reactor
Empty broth from harvest vessel into sample bottle
Print cycle number just completed
Return to start of dosing cycle

the medium. Initially a cycle time in excess of the minimum doubling time obtained during exponential batch growth is selected since phasing is likely to slow growth to some extent.

4.2.2 Results

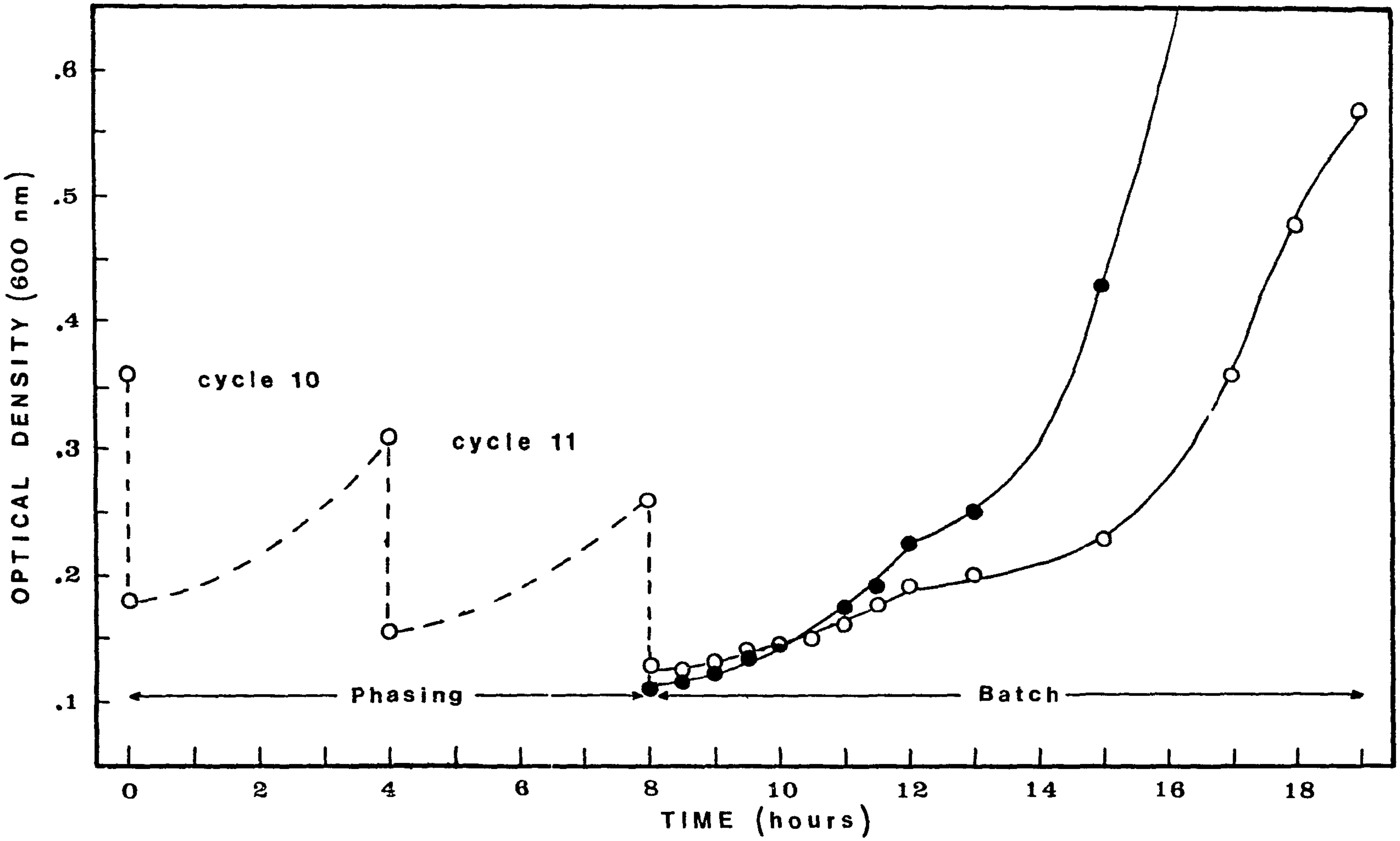
The first experiments were conducted with a medium that was limited by glucose at a concentration of 2.5 g/L. Batch culturing had indicated that a minimum biomass doubling time of about 2.3 hours could be expected with a manganese concentration of 3 x 10^{-4} molar and 2.9 hours with the manganese decreased to 5 x 10^{-5} molar. Initially the higher manganese concentration was used in conjunction with 2.5 g/L of glucose and an imposed nutrient cycle of four hours duration. It was judged that this cycle time would be amply sufficient to permit biomass doubling. This experiment was beset with many startup problems including excessive foaming, poor volume control and large variations in the biomass concentration. The experiment was concluded after 92 cycles.

The next experiment under carbon limitation was designed to investigate the effects of manganese. The manganese concentration was reduced by 83% from 3 x 10^{-4} molar to 5 x 10^{-5} molar while maintaining a 4-hour cycle time and a reduced glucose concentration (1.25 g/L). Analysis of the samples obtained at the end of each cycle indicated that the cells were unable to double during the four-hour imposed cycle time, and washout of the biomass was occurring. This is illustrated in Figure 18 by showing the change in optical density of the broth for cycles 10 and 11, followed by a batch culture. Because the cells had been washing out, there were excess nutrients available in the medium to support more than a single biomass doubling. Therefore, the batch culture was begun by simply stopping the four-hour dosing and harvesting cycles. The batch was operated in duplicate in a secondary reactor, using the harvested broth from the primary reactor at the end of cycle 11 as the inoculum, and with the addition of extra manganese in the medium. These results revealed some important phenomena. Batch culturing had indicated that with a manganese concentration of 5 x 10^{-5} molar, the minimum doubling time obtainable is about 2.9 hours during the logarithmic growth phase. Based on this, a cycle time of four hours should have been sufficient for biomass doubling. However, one effect of the period of nutrient dosing is apparently to reduce the specific growth rate when compared to the maximum specific growth rate in batch culture, otherwise cell washout would not have occurred. The effects of an increase in the manganese concentration are clearly seen, explaining the stability that was achieved with 3 x 10^{-4} molar manganese and a four hour cycle time. The most surprising phenomenon is the discontinuity in growth that occurs fours hours into the batch culture. It is important

Figure 18

Continuous Phasing with Four-hour Imposed Nutrient Cycles with Carbon Limitation

Shown is the change in biomass concentration, as monitored by optical density at 600 nm, for two consecutive four-hour nutrient cycles followed by a batch culture, at two concentrations of manganese, 3 x 10^{-4} molar (●) and 5 x 10^{-5} molar (○).


OPTICAL DENSITY (600 nm)
.6
.5
.4
.3
.2
.1
cycle 10
cycle 11
Phasing
Batch
0
2
4
6
8
10
12
14
16
18
TIME (hours)

to note that at this point nothing was removed or added to the culture. Yet, it appears that the cells were "expecting" another nutrient cycle and an intermediate lag period occurred prior to the logarithmic growth. Intermediate periods of no growth were also observed during the operation of consecutive batches (Figure 13).

The effects of an excessively long nutrient cycle on the stability of the system are illustrated in Figure 19. In this case, two 6-hour cycles are shown with two different manganese concentrations in the medium. Again, the faster specific growth rate is obtained with the higher manganese concentration (3×10^{-4} molar). However, after the maximum biomass is attained at an optical density of 1.0, there is a rapid decrease in the biomass. This was also observed in conventional batch cultures under carbon limitation and is presumably a result of cell lysis. Therefore, an instability is created if an excessive time period occurs between exhaustion of the limiting nutrient and dosing of the fresh nutrients.

Table 3 summarizes the minimum biomass doubling times obtained under carbon limitation as affected by manganese concentration and the technique of cell culturing that was employed. As the cycle time is increased, the specific growth rate approaches that which was obtained in batch culture.

All subsequent phasing experiments were conducted with a nitrogen-limited medium. This change was implemented due

Figure 19

Six-hour Nutrient Cycles with Carbon Limitation

Shown are the changes in biomass concentration, as monitored by optical density at 600 nm, for two consecutive 6-hour nutrient cycles at two concentrations of manganese, 3×10^{-4} molar (●) and 5×10^{-5} molar (○).

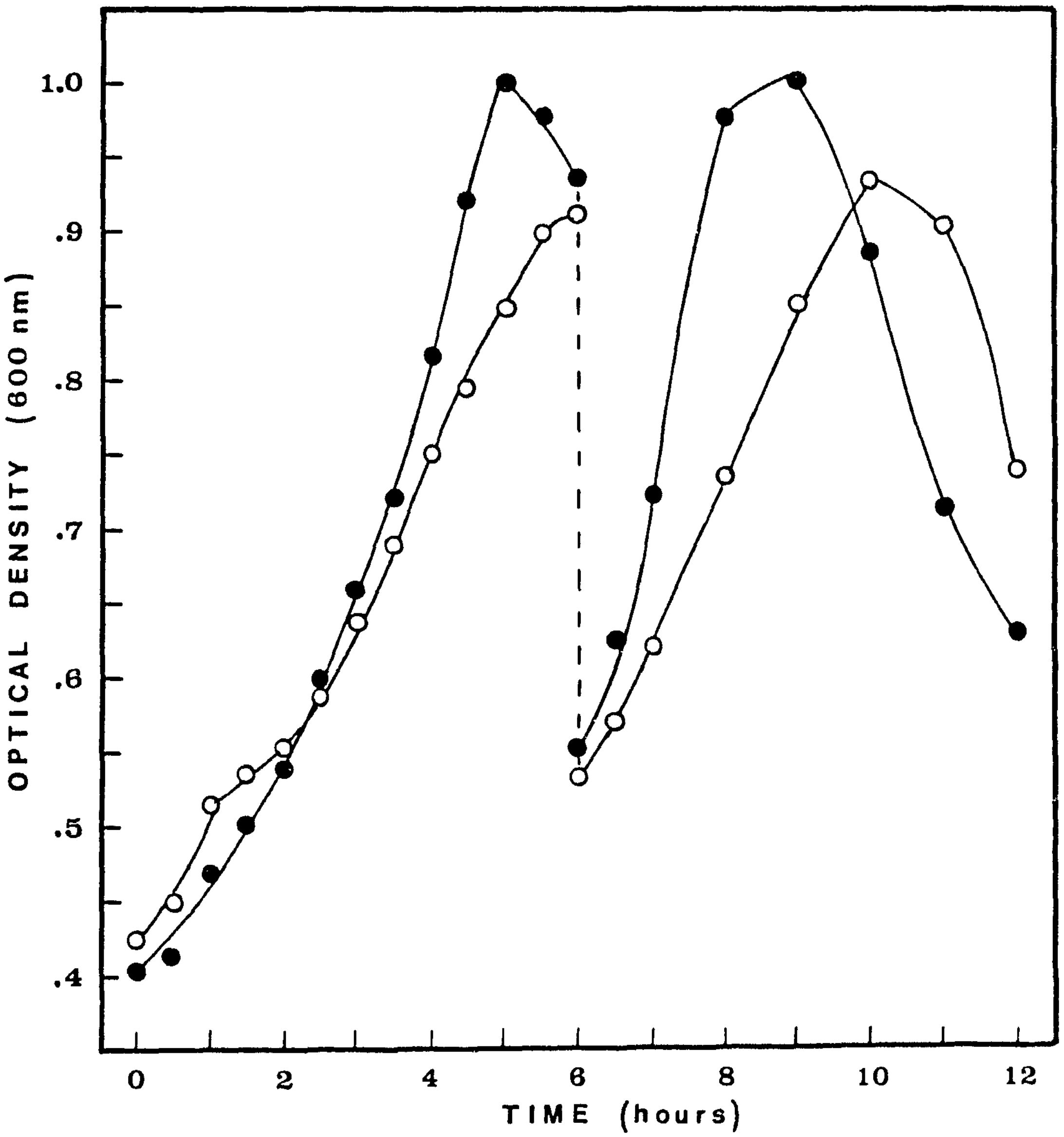

OPTICAL DENSITY (600 nm)
1.0
.9
.8
.7
.6
.5
.4
0
2
4
6
8
10
12
TIME (hours)

Table 3

Effects of the Cell Culture Technique on Specific Growth Rate

Technique	Doubling times (hrs)*	
	0.5×10^{-4} molar Mn	3.0×10^{-4} molar Mn
Batch	2.9	2.3
4-hour phasing	5.4	2.7
6-hour phasing	4.9	2.3

* calculated from changes in the optical density of the broth during the logarithmic growth phase of batch culture, or between the start of a phasing cycle and the time at which the optical density has doubled.

to the reduction in cell lysis that occurs following nitrogen exhaustion (when compared to carbon) and the ease in analyzing the nitrogen concentration in the broth.

With B. subtilis growing in a 0.002 molar $(NH_4)_2HPO_4$ limited medium, the fastest biomass doubling time in conventional batch culture was about 2.4 hours, therefore, 4.0 hours was selected as the first cycle time. The results of this first continuous phasing experiment in which level probes were used to measure volumes, are presented in Figures 20 to 23. The first 58 generations of cells were monitored by analyzing the broth harvested at the end of each cycle for surface tension, dry weight and viable cell count (Figure 20).

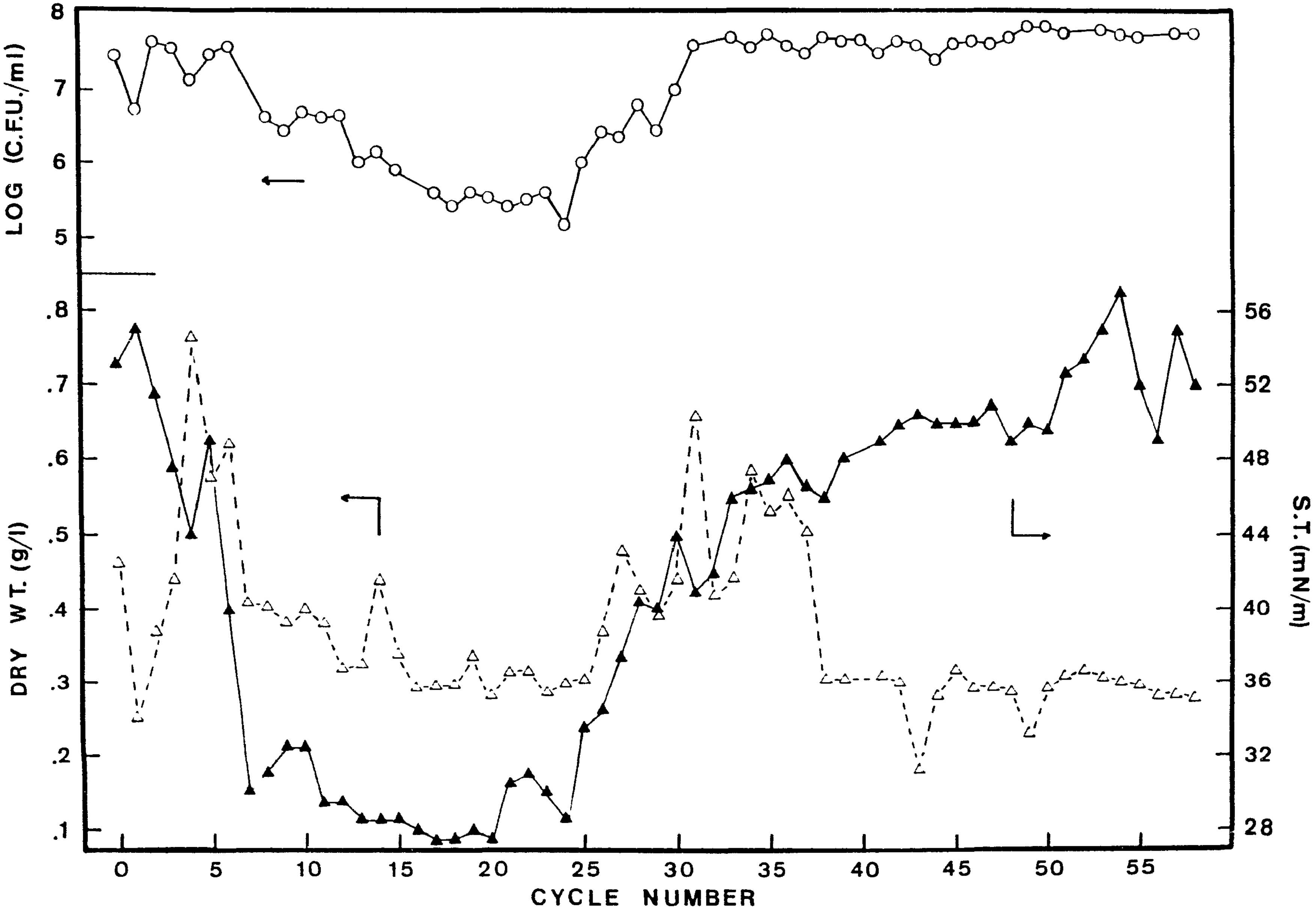
These results show that the system was not stable. There is considerable variation in performance throughout the experiment. This was also found under carbon limitation. The changing response of the cell population could be divided into four phases. In the first phase, consisting of about 7 generations, there is a rapid decline in both cell number and surface tension. This was followed by a relatively constant phase, lasting about 17 generations, during which surfactant production was high. Then, beginning at cycle 25, both cell number and surface tension increase before plateauing around cycle 35. This pattern of response was apparently an adaptation to the periodic nutrient supply and reveals changes in the metabolism of each succeeding generation of cells.

Second stage studies (as described in Section 1.4) were

Figure 20

Continuous Phasing with Four-hour Imposed Nutrient Cycles with Nitrogen Limitation

Shown are the changes in colony forming units or viable cell count (○), dry weight of biomass (△) and surface tension (▲) over 58 consecutive nutrient cycles.


LOG (C.F.U./ml)
8
7
6
5
DRY WT. (g/l)
.8
.7
.6
.5
.4
.3
.2
.1
S.T.(mN/m)
56
52
48
44
40
36
32
28
0
5
10
15
20
25
30
35
40
45
50
55
CYCLE NUMBER

conducted during cell cycles 41, 81 and 100 to investigate possible trends during single generations. These cell cycles correspond to a period after the best surfactant production and the surface tension was still gradually increasing. From Figure 21 it can be seen why this is occurring. The nitrogen was exhausted at approximately three and one half hours, after which there was a rapid increase in surface tension from about 43 to 50 mN/m at the end of the four hour cycle. This increase continued into post cycle and then finally decreased again at five and a half hours to 51 mN/m. Forty generations later, during cycle number 82 (Figure 22), the surface tension follows the same pattern, however, in this case the point of rapid increase has shifted into post cycle and would, therefore, not affect subsequent generations. Although nitrogen was exhausted at approximately the same time, growth, as monitored by optical density, stopped at about two and a half hours. Cell number increased only slightly until the two and a half hour point when a rapid cell doubling occurred. This indicates that a relatively high degree of division synchrony had been attained. By the 100th generation (Figure 23) the surface tension was essentially constant at an elevated value, between 56 and 58 mN/m, throughout the cell cycle and two hours into post cycle. The nitrogen was exhausted by two and a half hours, thus starving each generation for about one and a half hours. From Figure 20 it can be seen that the viable cell count (CFU/mL) correlates

Figure 21

Four-hour Nutrient Cycle Number 41

Shown are changes in the nitrogen (□), surface tension (▲), biomass (●) and viable cell count (○) as they relate to the 41st consecutive four-hour nutrient cycle.

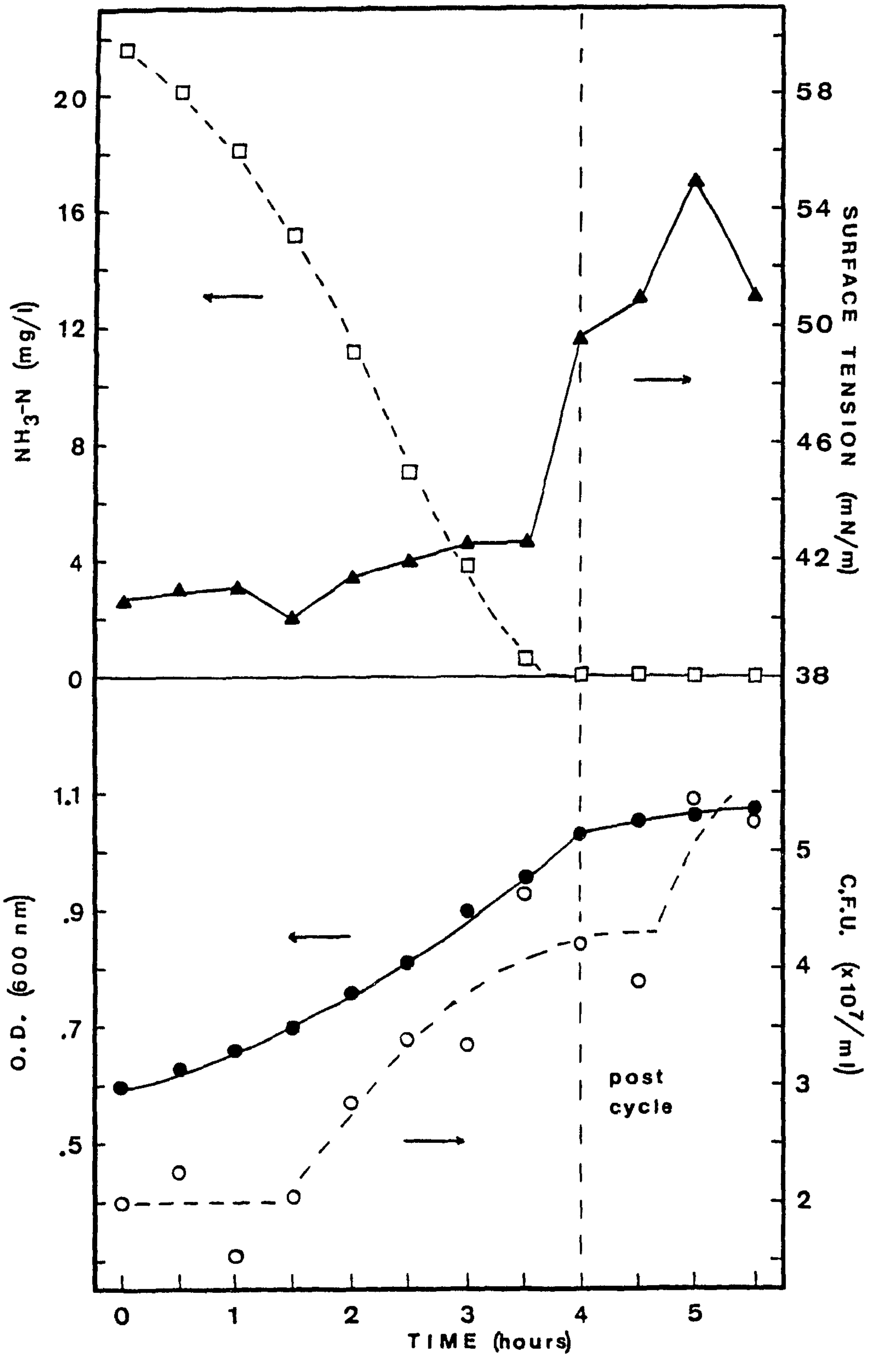
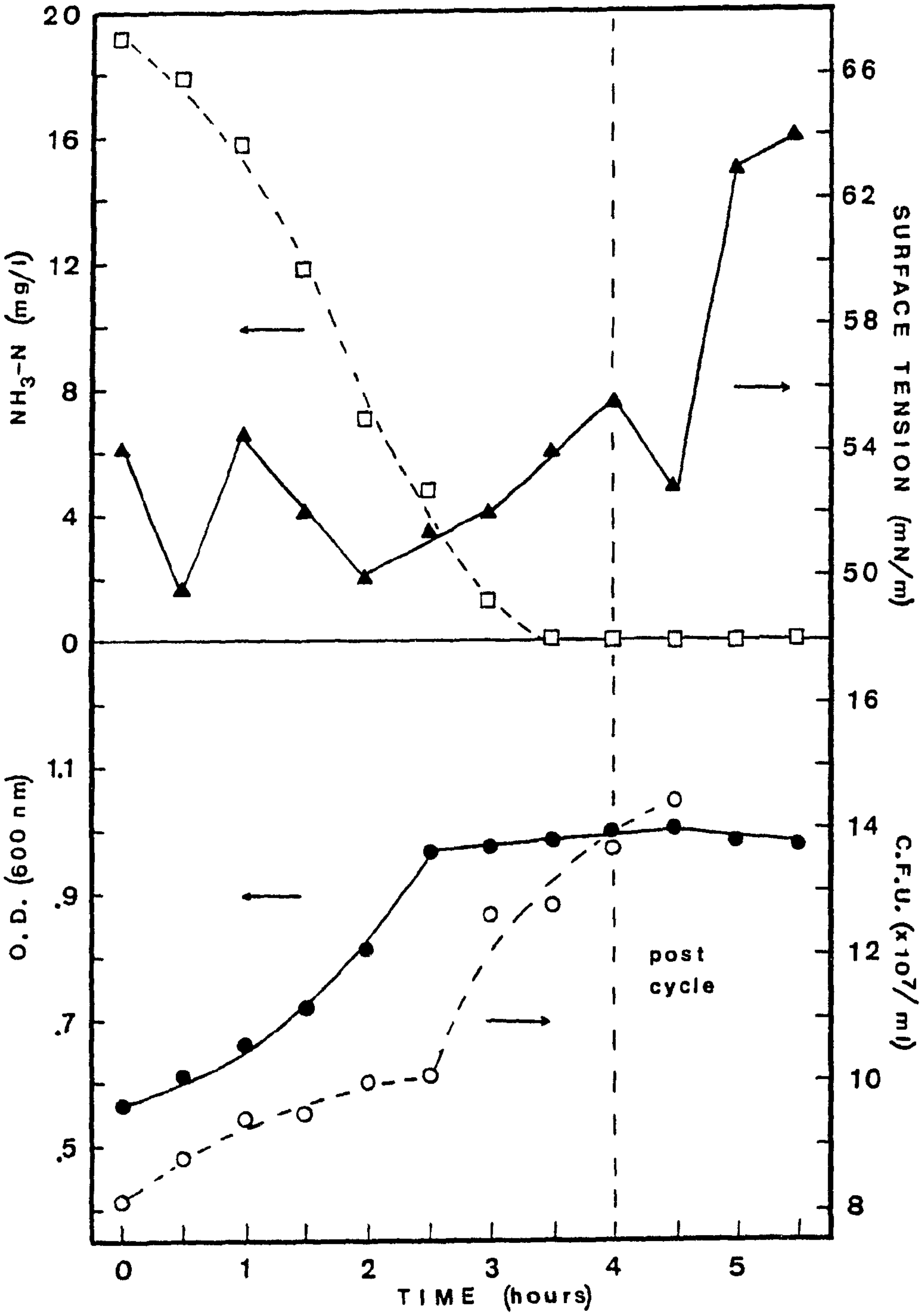

NH$_3$-N (mg/l)
20
16
12
8
4
0
SURFACE TENSION (mN/m)
58
54
50
46
42
38
O.D. (600 nm)
1.1
.9
.7
.5
C.F.U. (x10^7/ml)
5
4
3
2
post cycle
0
1
2
3
4
5
TIME (hours)

Figure 22

Four-hour Nutrient Cycle Number 82

Shown are changes in the nitrogen (□), biomass (●), surface tension (△) and viable cell count (○) as they relate to the 82nd consecutive four-hour nutrient cycle.

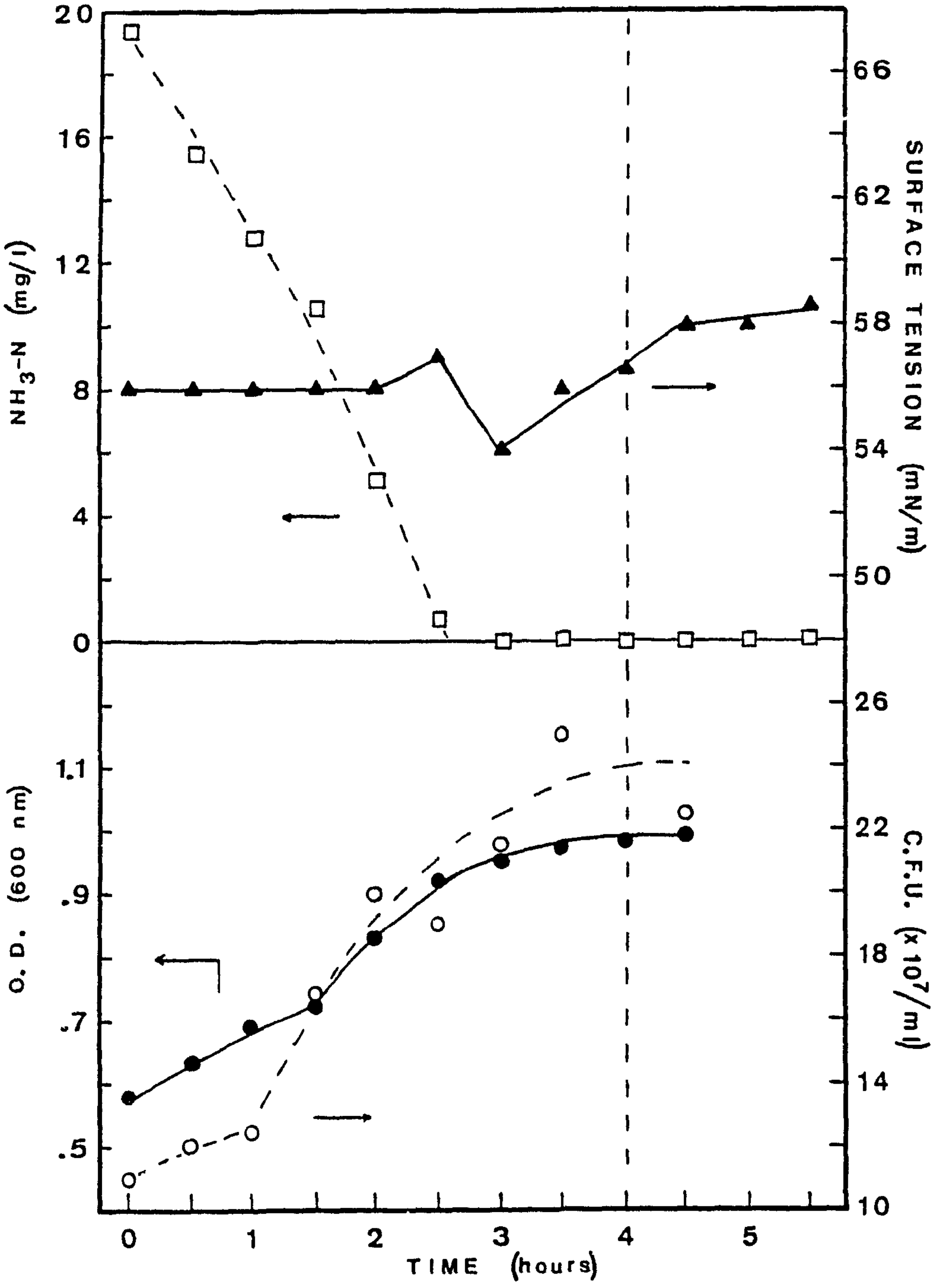


NH$_3$-N (mg/l)
20
16
12
8
4
0
SURFACE TENSION (mN/m)
66
62
58
54
50
O. D. (600 nm)
1.1
.9
.7
.5
C.F.U. (x10^7/ ml)
16
14
12
10
8
post cycle
TIME (hours)
0
1
2
3
4
5

Figure 23

Four-hour Nutrient Cycle Number 100

Shown are changes in nitrogen (□), surface tension (▲), biomass (●) and viable cell count (○) as they relate to the 100th consecutive four-hour nutrient cycle.



20
16
12
8
4
0
NH$_3$-N (mg/l)
1.1
.9
.7
.5
O. D. (600 nm)
66
62
58
54
50
SURFACE TENSION (mN/m)
26
22
18
14
10
C.F.U. (x 10^7/ml)
0
1
2
3
4
5
TIME (hours)

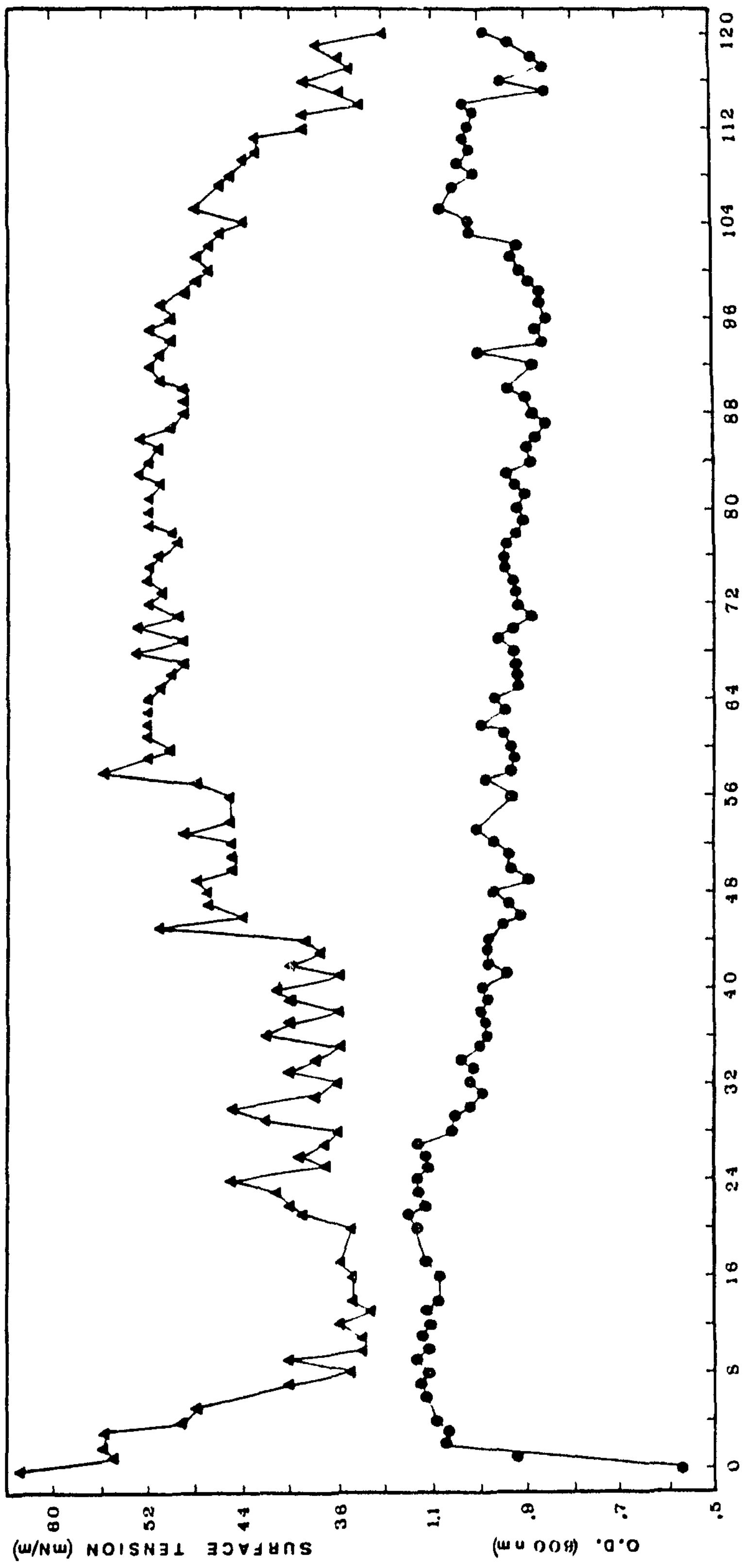
well with surface tension but not dry weight. This large change in cell count over the course of the experiment may have been a result of the sensitivity of the cells to lyse when subjected to a nutrient limitation in the presence of surfactin. Surfactin has been shown to be a powerful lytic agent by Arima et al. (1968) and Bernheimer and Avigad (1970). As the concentration of surfactin decreased following cycle 35, the number of cells that were able to survive the plating procedure may have increased significantly. Therefore, the change in viable cell count over successive cycles may have been an artifact of the plating procedure and is more a reflection of the levels of surfactin present in the broth.

The next continuous phasing experiment utilized the electronic balance and computer control of dosing and harvesting volumes. The computer also provided the flexibility of fractional cycle times as opposed to only integer multiples of an hour which were available with the timer. Figure 24 illustrates the response of the population to a 200 minute imposed cycle time over 120 consecutive cell generations. The same overall pattern in surface tension was observed, however, there was more instability and generally higher surface tension than obtained with a four hour imposed cycle. This experiment was continued past 100 cell generations and, of possible significance, was a second decreasing trend in surface tension after cycle 111, eventually dropping to 32 mN/m at cycle 120.

Figure 24

Continuous Phasing with 200-minute Imposed Nutrient Cycles

Shown are changes in biomass concentration, as monitored by optical density at 600 nm (●) and surface tension in the broth (▲) for 120 consecutive nutrient cycles of 200 minute duration.


SURFACE TENSION (mN/m)
60
52
44
36
O.D. (600 nm)
1.1
.9
.7
.5
CYCLE NUMBER
0
8
16
24
32
40
48
56
64
72
80
88
96
104
112
120

As previously stated, the fastest doubling time obtained in conventional batch culture with this same medium was about 2.4 hours or 145 minutes. It was, therefore, possible that the cycle time could be further reduced from 200 minutes in an attempt to increase stability and consistency between cell generations. A cycle time of 160 minutes was selected, however, as illustrated in Figure 25, relatively rapid washout of the cells occurred. The dry weight decreased from about 0.23 to 0.04 grams per liter in 16 generations. Therefore, this effect of the nutrient cycling on the maximum specific growth rate seemed to occur under either carbon or nitrogen limitations.

The ammonia nitrogen concentration increased during the 160-minute cycling from zero to 37 mg per liter and the surface tension oscillated between 42 and 54 mN/m. The washout was quickly reversed by increasing the cycle time from 160 minutes to 180 minutes. The biomass stabilized to about 0.25 grams per liter within 18 generations, corresponding to the point of full nitrogen utilization. It appeared, therefore, that the shortest cycle time that would sustain the biomass was between 160 and 180 minutes.

The next experiment utilized a 180 minute imposed cycle time, which was apparently close to the maximum specificgrowth rate under phasing conditions. Being close to the maximum would perhaps result in good stability as a result of minimal starvation or metabolic stress on the cells. The results from

Figure 25

Cell Washout During 160-minute Imposed Nutrient Cycles

Shown are changes in surface tension (▲), residual nitrogen concentration in the broth (□) and dry weight of biomass (△) while cycling every 160 minutes, until cycle 18, at which point the cycles were extended to every 180 minutes.

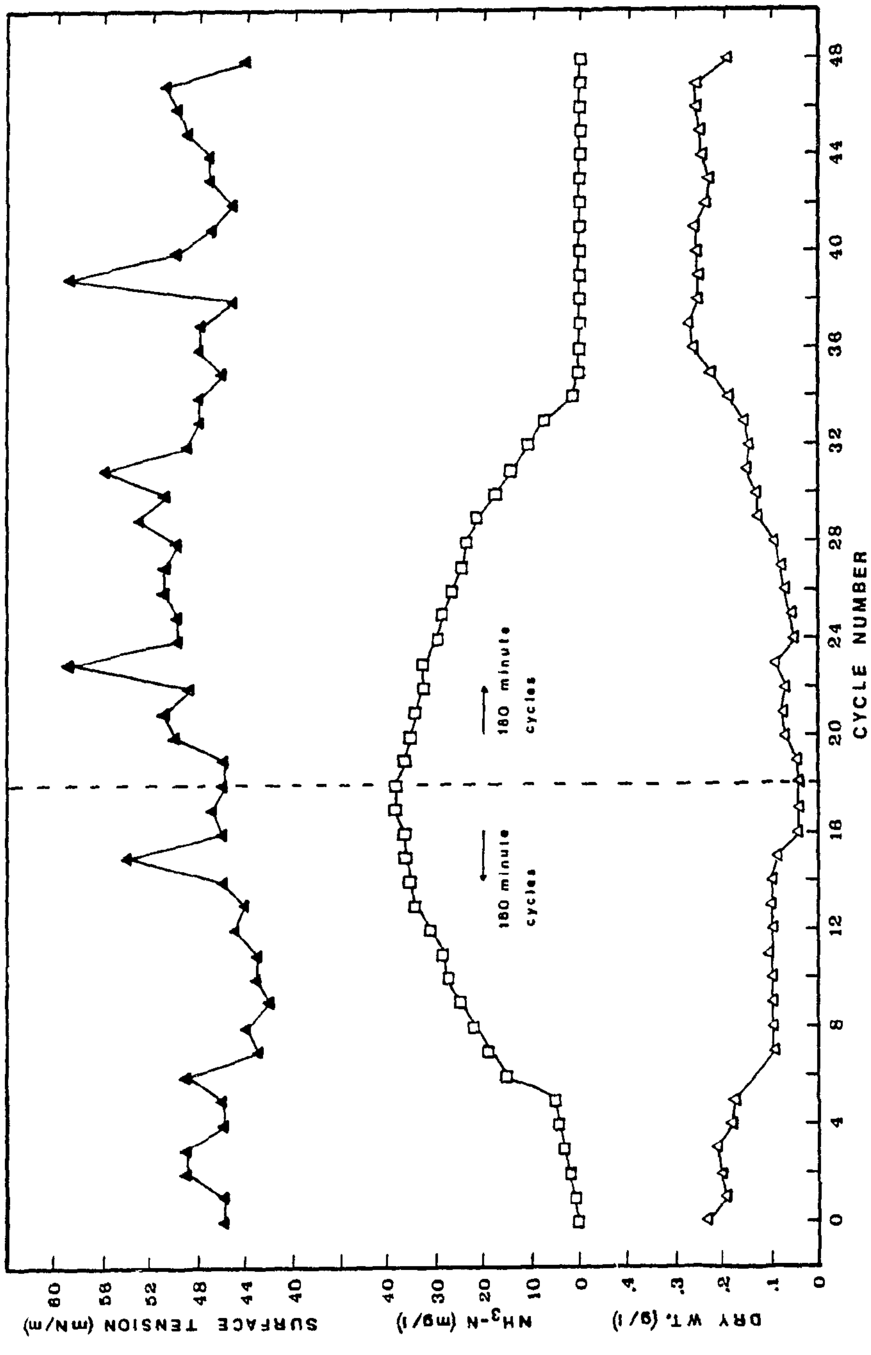
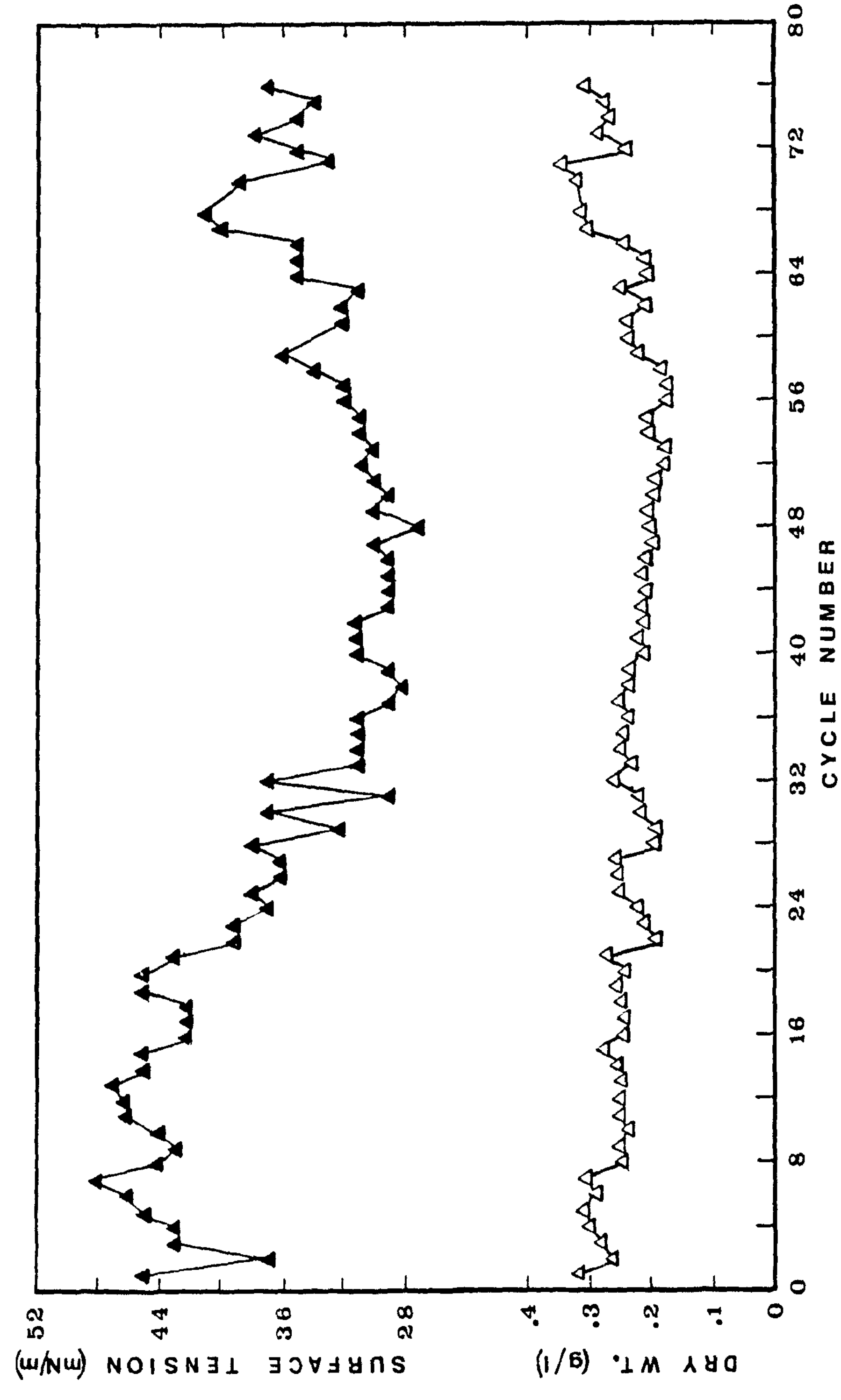

SURFACE TENSION (mN/m)
60
56
52
48
44
40
NH$_3$-N (mg/l)
40
30
20
10
0
DRY WT. (g/l)
.4
.3
.2
.1
0
180 minute cycles
180 minute cycles
CYCLE NUMBER
0
4
8
12
16
20
24
28
32
36
40
44
48

Figure 26

Continuous Phasing with 180-minute Imposed Nutrient Cycles

Shown are the changes in surface tension in the broth (▲) and dry weight of biomass (△) for consecutive nutrient cycles of 180 minute duration.

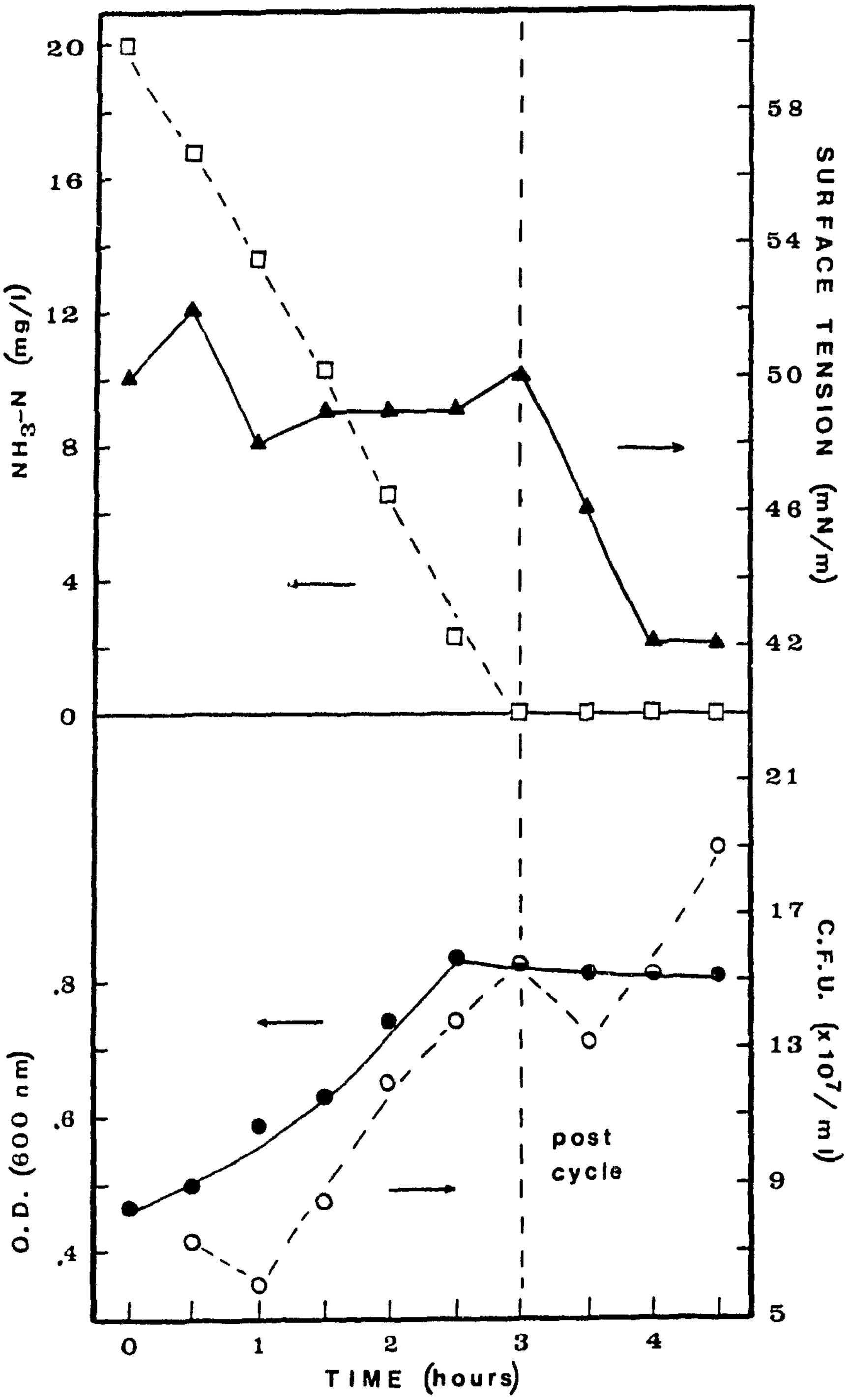


SURFACE TENSION (mN/m)
52
44
36
28
DRY WT. (g/l)
.4
.3
.2
.1
0
CYCLE NUMBER
0
8
16
24
32
40
48
56
64
72
80

Figure 27

Three-hour Nutrient Cycle Number 78

Shown are data obtained from a second stage experiment of changes in nitrogen (□), surface tension (▲), biomass (●) and viable cell count (○) for the 78th consecutive three-hour nutrient cycle.


20
16
12
8
4
0
NH$_3$-N (mg/l)
58
54
50
46
42
SURFACE TENSION (mN/m)
21
17
13
9
5
C.F.U. (x10^7/ml)
.8
.6
.4
O.D. (600 nm)
post
cycle
0
1
2
3
4
TIME (hours)

the 180 minute cycles are presented in Figures 26 and 27. The system did appear to have relatively good stability, although, there was still evidence of changing metabolic performance. Surfactant production was maintained between cycles 33 and 57 with the surface tension averaging about 30 mN/m and the biomass about 0.2 grams per liter. A second stage experiment revealed the cell cycle events during cell generation 78 (Figure 27). There is a sharp decrease in surface tension following the time of nutrient exhaustion (3 hours), however, this effect would not be seen in successive cell generations because it occurs in post cycle.

The results from the three different imposed cycle times are summarized in Table 4. It would appear that the most sustained surfactant production was obtained with the 180 minute cycle time, the cycle time that apparently corresponded closest to the doubling time of B. subtilis under continuous phasing conditions with this specific medium. However, in all cases the cell population eventually ceased producing significant quantities of surfactant, apparently undergoing a metabolic adaptation that required a number of generations to be fully manifest.

4.2.3 Discussion

The conventional method of continuous phasing was used in an attempt to relate surfactant production to the cell

Table 4

Comparison of Imposed Cycle Times

Cycle Time (min)	Production Period* (cycles)	Average Surface** Tension (mN/m)
240	18	29
200	13	35
180	34	30

* number of nutrient cycles during which significant quantities of surfactin were produced, as monitored by the surface tension of the broth.

** average surface tension of the harvested broth during the production period of surfactin.

cycle and, with this insight, to optimize the process. Although a full understanding of the environmental factors involved in promoting surfactant production was not achieved, the technique did reveal several important phenomena that provided a rational background for the subsequent refinement of the technique. Continuous phasing revealed the importance of the imposed cycle time, the metabolic adaptability of the culture and an apparently critical cell cycle control point that occurs around the time of nutrient exhaustion in the broth. This could only have been revealed by continuous phasing and second stage studies that extend into post cycle events. Dawson (1970, 1972) has documented the importance of this transition in the metabolism of _Candida_ _utilis_. These three phenomena can be related through the common factor of relative starvation time, or the period at the end of cell growth that the cell must survive when an essential nutrient is no longer available in the medium. This can be considered an environmental stress that prevents growth of new cells until the time of fresh nutrient addition. Associated with this stress two apparently opposite effects were observed, either a decrease or increase in surface tension. Which effect occurred during any particular generation could not be predicted without following a pattern that was manifest over many successive generations. The general pattern was consistent, regardless of the imposed cycle time, revealing an initial increase in surfactant production followed by an apparent adaptation, as reflected by a decrease in production. Apparent changes in cell cycle behaviour over successive generations of B. subtilis have also been observed by Maruyama et al. (1977) using continuous phasing.

The selected period of nutrient cycling was a critical experimental parameter. In batch culture only the last generations of cells are actually nutrient limited from the perspective of preventing growth, although, all generations may have limitations on growth rate as a result of the specific composition of the medium. In continuous phasing every generation of cells is subjected to an absolute nutrient limitation and a subsequent "starvation" period prior to the start of the next cycle. This drastic discontinuity in the nutrient environment that occurs at the time of nutrient dosing, apparently requires some metabolic adjustment before growth proceeds. This is evident from the experiments conducted under either carbon or nitrogen limitation. Washout of the cells occurred with an imposed period of nutrient cycling in considerable excess of the minimum cell doubling time obtained in batch culture. Further evidence is provided during the four hour cycling with carbon limitation at low manganese concentrations. Even though the cells were being washed out as a result of an effective doubling time of about 5.4 hours, (calculated from the decrease in biomass from cycle to cycle) reverting to batch culture revealed that the cells had been "programmed" to expect a discontinuity in the nutrient environment every four hours. The programmed reaction of the cell population was to enter another lag phase even though no discontinuity in the environment occurred. This emphasizes the dramatic importance of an abrupt change in the nutrient environment for controlling cellular metabolism, effects that can apparently be manifest over successive cell generations.

Interpretation of the cell cycle events as studied in individual generations, or apparent adaptive responses manifest over many successive generations should account for the interaction between the imposed nutrient cycle and the dynamics of the cell cycle. The most obvious ramification of changes to the imposed nutrient cycle is the effect on the duration of the period of nutrient starvation. This period affects subsequent generations as revealed by changes in specific growth rate and surfactant production. However, the systematic study of this phenomenon is difficult due to interactive effects of alterations to the imposed period of nutrient dosing, and the lack of any rationale for selecting a base case. Therefore, what is required is a reversal of the control strategy such that the period of nutrient dosing becomes a dependent variable, dependent on the cellular metabolism. This can be accomplished by incorporating a feedback circuit to control the nutrient dosing based on the metabolic requirements of the cells. Thus, as the requirements of the cells change over successive generations, the system will automatically respond ensuring a constant relationship between the nutrient cycle and the cell cycle.

4.3 Continuous Phasing with Feedback Control

4.3.1 Procedure

Unfortunately the conventional control of continuous phasing requires that a cycle time be selected and, therefore, imposed on the population rather arbitrarily. It soon becomes clear if the cycle time is too short, however, "excessive" cycle times cannot be determined without some previous knowledge of the range of metabolic flexibility and effects of starvation, and these are the very parameters that require investigation.

The imposition of a particular cycle time cannot be criticized because it results in an "unnatural" metabolic condition (Edwards 1981) but rather on the basis of the great difficulty of interpretation because of the lack of a base case. This problem has been overcome by modifying the control strategy such that no specific cycle time or cell doubling time is assumed. It is no longer an independent experimental variable. This was accomplished by noting the very close correlation between the concentration of dissolved oxygen and the exhaustion of the limiting nutrient. Following exhaustion of the limiting nutrient, there is a rapid increase in the level of dissolved oxygen, presumably as a result of a declining respiration rate. This effect is illustrated in Figures 10 and 11. The computer programme used for the control of the phasing period was modified to monitor the dissoved oxygen concentration such that cycling of the nutrients would be controlled by the respiration rate of the cells. In this way, an indirect measurement of the precise time of nutrient exhaustion was achieved for each generation of cells. The relationship between the cell growth and nutrient dosing was constant for each successive generation. Any change in the growth rate of the cells resulted in an immediate compensation in the period of nutrient dosing.

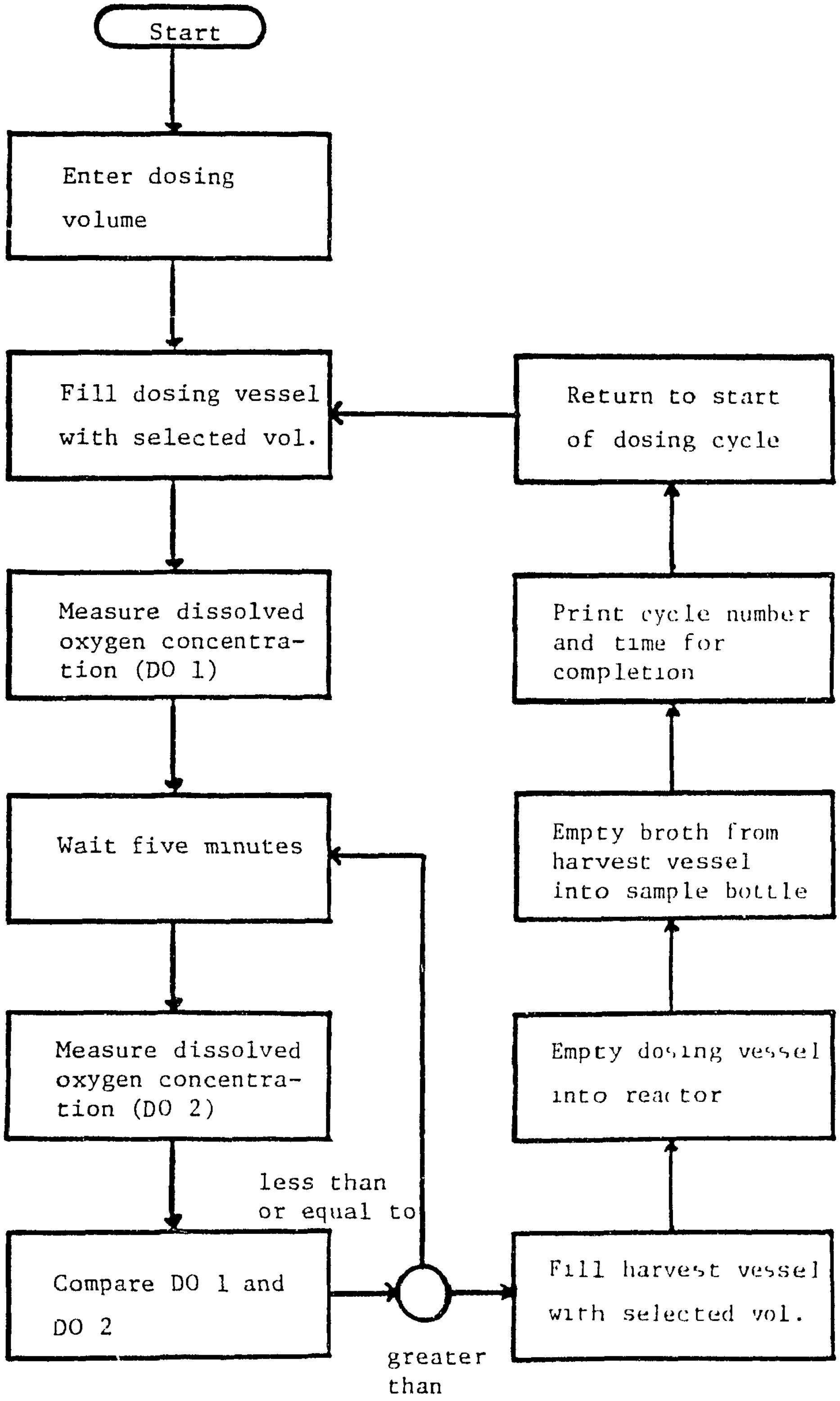
The inherent stability of the system, when controlled with this feedback strategy, opened up many new possibilities for experimentation. There was no longer the possibility of cell washout, regardless of the composition of the medium. No prior knowledge of specific growth rate was required, since no specific period of nutrient dosing is imposed on the cells. The specific growth rate during continuous phasing is now a reflection of the dynamic relationship between the cells and the nutrient environment, without the complication of an inflexible environmental stimulus. It is now truly an interactive system with the control based only on the relationship between the cell population and the nutrient environment.

The logic of the computer programme is presented in Figure 28. In Version 3, the only operator selected variable

Figure 28

Flow Chart for Control of Continuous Phasing Using Feedback

Shown is a simplified flow chart which forms the basis for computer programmes Versions 3 to 12.


Start
Enter dosing volume
Fill dosing vessel with selected vol.
Measure dissolved oxygen concentration (DO 1)
Wait five minutes
Measure dissolved oxygen concentration (DO 2)
Compare DO 1 and DO 2
less than or equal to
greater than
Fill harvest vessel with selected vol.
Empty dosing vessel into reactor
Empty broth from harvest vessel into sample bottle
Print cycle number and time for completion
Return to start of dosing cycle

is the dosing and harvesting volume, usually entered as one half of the total working volume in the reactor. The important new feature is the internal loop that periodically monitors the concentration of dissolved oxygen, in Version 3 every five minutes. Subsequent versions use much more frequent measurements and an averaging function. The computer receives this information via the analogue to digital conversion in the HP3421A Data Acquisition unit. The programme compares successive values of dissolved oxygen until there is a change in oxygen usage such that the most recent measurement (DO2) is greater than the preceding value stored in memory as DO1. At this point a harvesting and dosing cycle is initiated to provide fresh nutrients to the cells. The computer sends data to the printer which records the cycle number, the total cycle time (biomass doubling time) and the minimum dissolved oxygen concentration that was measured. A complete listing of all the programmes written in HP Advanced Basic is included in Appendix C.

Table 5 summarizes several variations of this basic programme which include the following features:

i) The operator can select a period of nutrient starvation that will delay the dosing of fresh nutrients for a specific period of time following exhaustion of the limiting nutrient. This period will be the same for each successive generation of cells (Version 5).

ii) The operator can select a specific concentration of dissolved oxygen that will trigger the nutrient cycle. This allows operation without exposing the cells to any period of absolute nutrient limitation (Version 6).

iii) The programme automatically monitors the amount of foam collected in a special vessel attached to the air outlet. This gives a qualitative indication of the amount of surfactant produced (Version 7).

iv) The operator can select a maximum allowable nutrient cycle time. If this time is exceeded, the programme automatically switches to a different medium for subsequent dosing. This allows the effects of a gradual decrease in the concentration of a rate limiting nutrient to be tested without experiencing excessive doubling times (Version 8).

v) The operator can select a pattern for automatic switching of the medium supply between two containers. This allows one or more nutrients in the medium to be varied in concentration over succeeding generations of cells (Versions 9 and 10).

vi) The operator can select desired concentrations of 2 nutrients and the period for square wave cycling of one of these nutrients. This allows the testing of the response of the cell population to a square wave variation in the concentration of one of the nutrients (Version 11).

Table 5

Computer Programmes for Control of Nutrient Dosing

Version #	Features
1	Select nutrient cycle time and dosing volume.
2	Version 1 plus automatic volume correction.
3	Feedback control based on change in dissolved oxygen.
4	Version 3 plus 60 minute pause after cycling, and dissolved oxygen averaging function.
5	Version 4 plus selection of starvation period.
6	Feedback control based on minimum dissolved oxygen.
7	Version 5 plus measurement of foam production, and improvement on dissolved oxygen monitoring.
8	Version 7 plus selection of maximum cycle time, and automatic switching of medium supply but only 2 sample valves.
9	Version 7 plus selection of pattern for automatic switching of medium supply but only 2 sample valves.
10	Version 9 plus selection of desired nutrient concentrations (2) but only one sample valve.
11	Version 7 plus selection of desired nutrient concentrations (2), and period for square wave nutrient cycling, but only one sample valve.
12	Version 7 plus selection of desired nutrient concentration (1), and period for square wave nutrient cycling, 2 sample valves.

An important aspect of process optimization involves the selection of an appropriate medium for cell growth and product formation. With a bacterium such as B. subtilis there is essentially an infinite number of recipes that would support growth. Therefore, there is a need for a method that will allow the process engineer to test changes in the nutrient environment, without resorting to tedious, and generally unproductive, trial and error. The computer control programme (Versions 9 through 12) can provide a periodic variation in one or more of the nutrients that spans any number of cell generations. The phasing technique ensures that all successive generations are subjected to an identical environment, except with respect to the nutrient being tested. In addition, the feedback control maintains stability by automatically adjusting the period of nutrient addition to correspond to changes in the growth rate, as the cell population responds to the changing nutrient concentration. The adjustment required by the system is reflected by changes in the nutrient cycle, which is directly representative of the metabolic response of the cells. In this way, the dynamic response of successive generations of cells to a single nutrient can be rigorously characterized. Testing of several key nutrients will allow a methodical progression towards an optimal medium.

The basic medium used for the continuous phasing experiments with feedback control was the same as described in Section 2.9 and Table 1, with nitrogen in the form of diammonium phosphate as the limiting nutrient. All experiments were performed with 400 mL dosing and harvesting volumes with a 800 mL working broth volume in the reactor. This volume ratio ensures that if the biomass concentration (as monitored by optical density or dry weight) is constant at the end of consecutive cycles, that the biomass must be doubling during each cycle. Furthermore, if growth continues to the end of each cycle, then the perid of nutrient cycling (cycle time) will correspond to the reciprocal of the specific growth rate.

4.3.2 Results

4.3.2.1 Minimal starvation

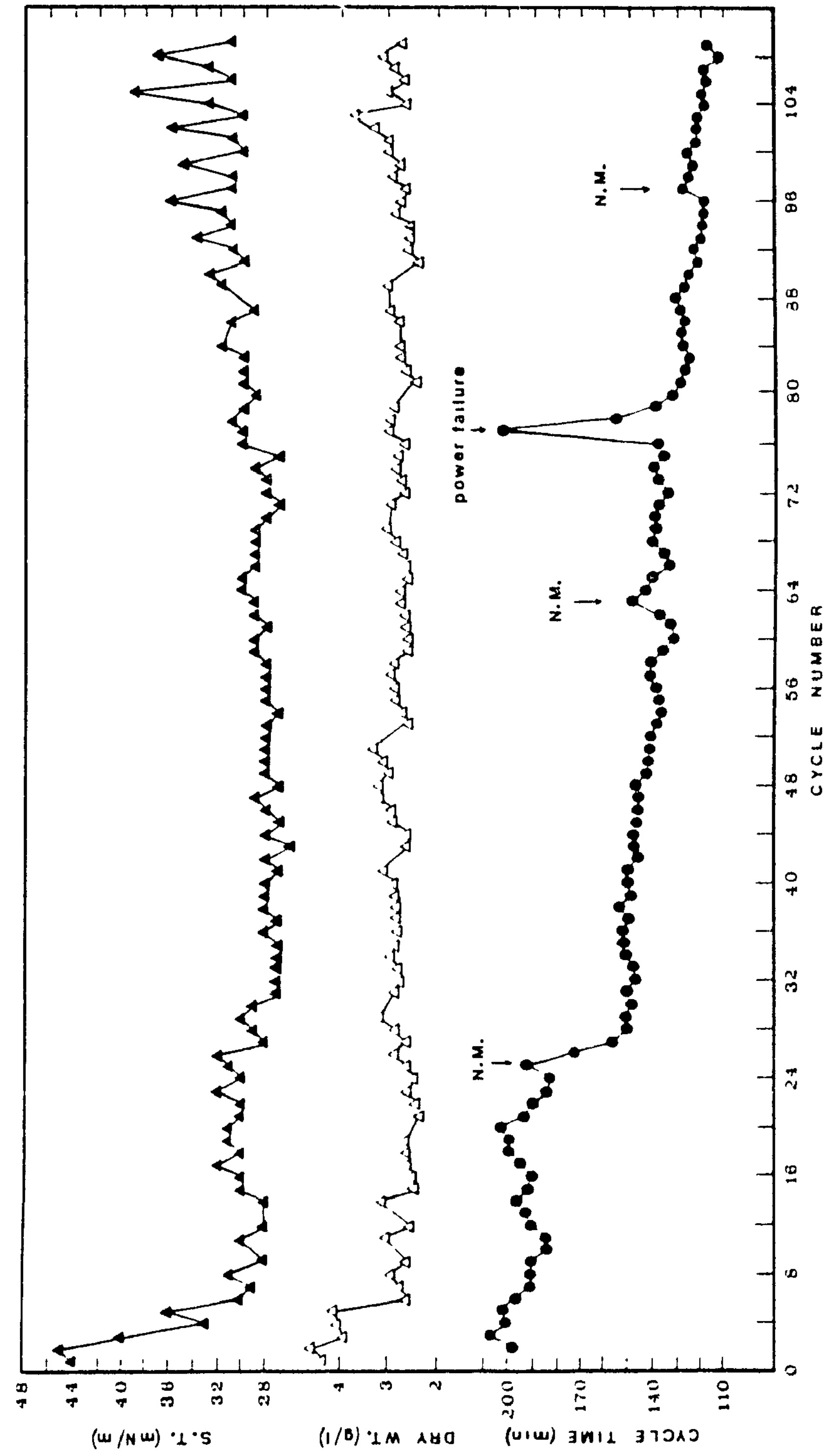
The first experiment incorporating the feedback control system was operated with the computer initiating a new nutrient cycle immediately after determining that the dissolved oxygen was increasing. This is referred to as "zero minute starvation", although, there is actually a short time that the nitrogen level in the medium is zero. The length of this time delay is a result of several factors: the metabolic lag between when the nitrogen is exhausted and the decrease in the respiration rate of the cells, the rate of oxygen transfer into the medium, the response of the probe, the period of dissolved oxygen sampling by the data acquisition unit and the time required to harvest the broth prior to dosing with fresh nutrient. When these factors are added together, the total time of nutrient exhaustion is between 5 and 10 minutes during a typical 120 minute cell cycle. Starvation is, therefore, defined as the time period when the growth limiting nutrient is exhausted in the medium, even though the cells may be using stored nutrients.

The results from an extended feedback phasing experiment with this minimal starvation period are presented in Figure 29. The surface tension at the end of the batch, or beginning of phasing, was 44 mN/m and by cycle 6 had decreased to 30 mN/m. This initial increase in surfactant production in response to nutrient cycling was observed previously with a 4-hour imposed cycle time. Initially, the cycle time based on feedback was about 200 minutes, although, this decreased sharply to 150 minutes after a new batch of medium was prepared at cycle 26, followed by a slow decline over the next 80 generations to about 120 minutes. Between cycles 6 and 83 (77 generations of cells) the surface tension averaged below 30 mN/m. After cycle 83 an oscillation developed, where, every third cycle an increase in surface tension occurred, increasing to about 36 mN/m and decreasing back to 30 to 31 mN/m the following cycle. Throughout the entire experiment the system exhibited a high degree of stability both mechanically and biologically, with the biomass averaging 0.28 grams per liter, regardless of the specific growth rate (cycle time) or upsets such as a brief power failure. The experiment

Figure 29

Continuous Phasing with Feedback Control and Minimal Cell Starvation

Shown are changes in the surface tension of the broth (▲), dry weight of biomass (△) and nutrient cycle time (●) for 109 consecutive generations of cells. New batches of media were prepared at the cycles indicated by N.M.


S.T. (mN/m)
48
44
40
36
32
28
DRY WT. (g/l)
4
3
2
CYCLE TIME (min)
200
170
140
110
N.M.
N.M.
power failure
N.M.
0
8
16
24
32
40
48
56
64
72
80
88
96
104
CYCLE NUMBER

was finally terminated after 109 generations due to an extended power failure.

Two second stage experiments were conducted during cycles 14 and 90 (Figure 30). During both experiments the surface tension remained low throughout the cell cycle with maximum biomass corresponding to the time of nitrogen depletion. The decreased cycle time during cycle 90 is reflected by the rate of nitrogen depletion and decrease in cycle time from 196 to 126 minutes.

4.3.2.2 Controlled starvation

One of the advantages of being able to identify the point in the cell cycle at which nutrient exhaustion occurs, is that the computer can be programmed to delay dosing of fresh nutrient for any selected period of time. This introduces a controlled starvation period onto the end of each cell cycle. This strategy was used with Version 5 of the programme to test the effects of five different starvation periods: 120, 90, 60, 30 and zero minutes (actually about 5 minutes as discussed previously). Each starvation period was imposed on 14 successive cell generations, beginning with 120 minutes and decreasing by 30 minutes every 14 cycles. The results from the last 7 cycles subjected to each starvation period are summarized in Table 6 and data from all the cycles are presented in Figure 31.

Figure 30

Nutrient Cycles Numbers 14 and 90 from Continuous Phasing with Feedback Control

Shown are changes in surface tension, (△) and (▲), nitrogen, (□) and (■), and biomass as monitored by optical density at 600 nm, (○) and (●), for two nutrient cycles employing feedback control of the nutrient dosing. The 14th cycle is depicted by the open symbols and the 90th cycle by the closed symbols.

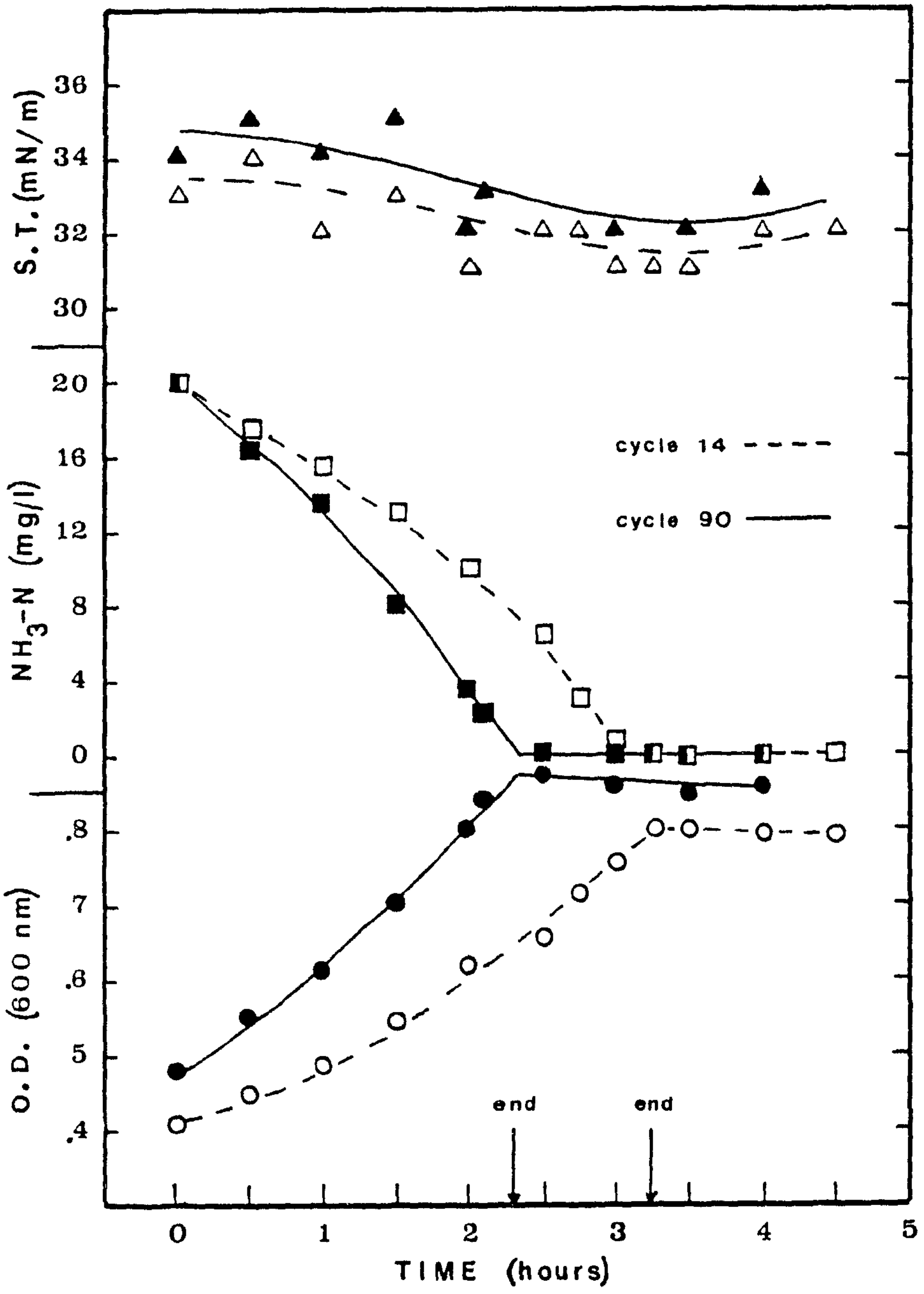

S.T. (mN/m)
36
34
32
30
NH_3-N (mg/l)
20
16
12
8
4
0
cycle 14
cycle 90
O.D. (600 nm)
.8
7
.6
5
.4
end
end
0
1
2
3
4
5
TIME (hours)

Table 6

Results from Controlled Starvation Using Feedback Control

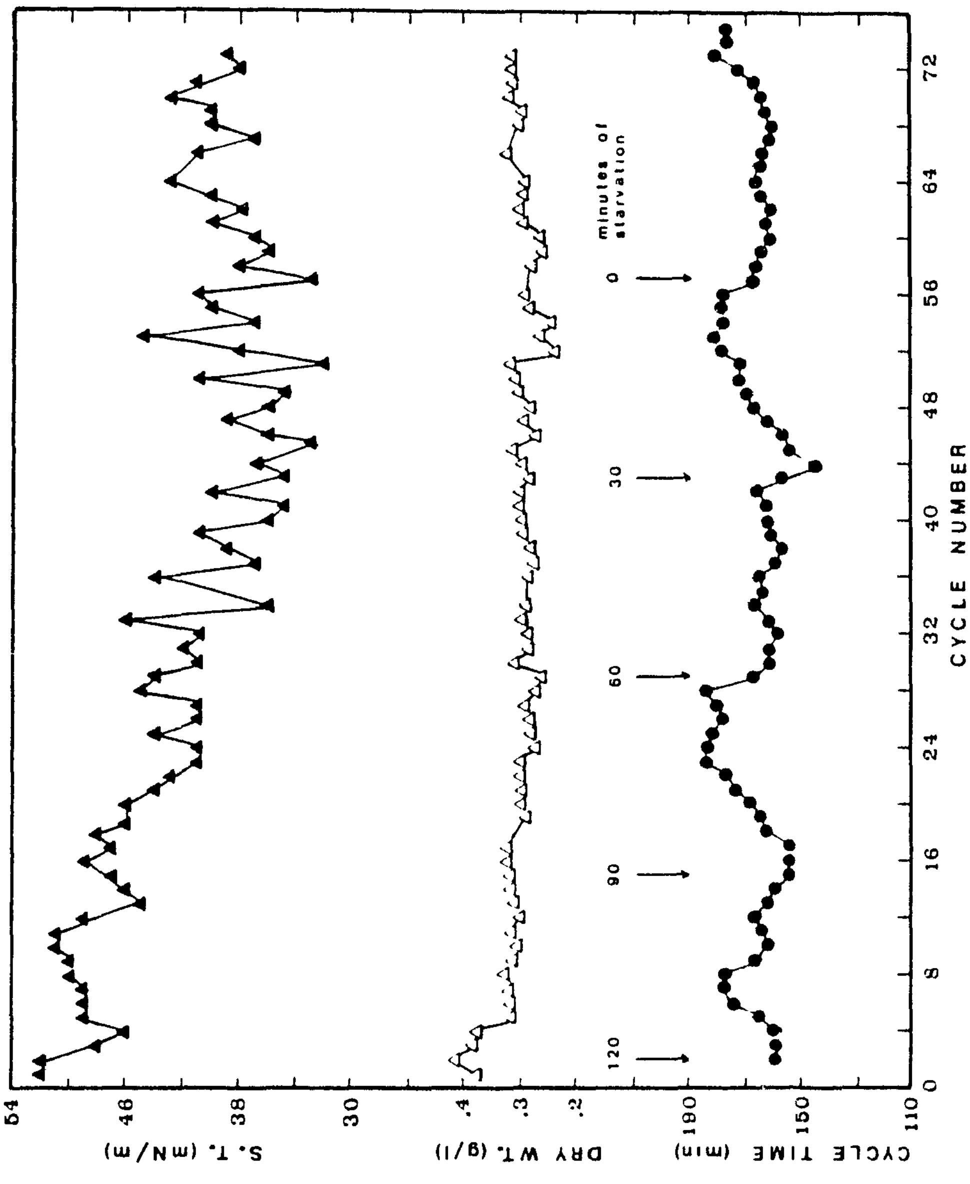
Starvation (min.)	Cycle Time* (min.)	Dry Weight* (g/L)	Surface Tension* (mN/m) (minimum/average)
120	166	0.32	45/49
90	182	0.29	41/42
60	162	0.29	35/39
30	178	0.28	32/39
0	164	0.31	37/41

* Averages from last 7 cycles of each period.

Figure 31

Continuous Phasing with Feedback Control of Cell Starvation

Shown are changes in surface tension in the broth (▲), the dry weight of biomass (△) and the net nutrient cycle time (●) as the starvation period at the end of the cycle is varied from 120 to 0 minutes.


S.T. (mN/m)
54
46
38
30
DRY WT. (g/l)
.4
.3
.2
CYCLE TIME (min)
180
150
110
CYCLE NUMBER
0
8
16
24
32
40
48
56
64
72
minutes of starvation
120
90
60
30
0

There appears to be a weak trend of decreasing surface tension with decreasing starvation, however, it is not very significant. This is due, perhaps, to the large instability in surface tension beginning during the 60 minute starvation cycles, with variations of 6 to 10 mN/m between successive cycles. There was also what appeared to be an irregular cyclic variation in cycle time, typically ranging between about 155 and 180 minutes during the course of each 14 cell generations. In all cases the growth rate was considerably slower than achieved in the previous phasing experiment with zero starvation. The biomass was very stable throughout the 72 cycles, averaging 0.3 grams per liter.

Figures 32 to 34 illustrate the results from three second stage experiments conducted during cycles 19, 36 and 66 corresponding to 90, 60 and zero minutes of added starvation respectively. Both cycles 19 and 36 show a decrease in surface tension between 210 and 240 minutes, after the time of nutrient exhaustion but before the end of the cycle. In contrast cycle 66 with zero minutes starvation shows an increase in surface tension which corresponds to the time of nutrient exhaustion and subsequent dosing of fresh nutrient.

It has been shown that the interpretation of population responses should account for possible metabolic effects that have occurred in past generations. Therefore, another starvation experiment was performed, but in this case phasing was started with zero minutes of added starvation. After an

Figure 32

Nutrient Cycle Number 19 with a 90-minute Starvation Period

Shown are the changes in nitrogen (□), surface tension (▲), biomass (●) and dissolved oxygen concentration (△) over the duration of a nutrient cycle with an added 90-minute starvation period.

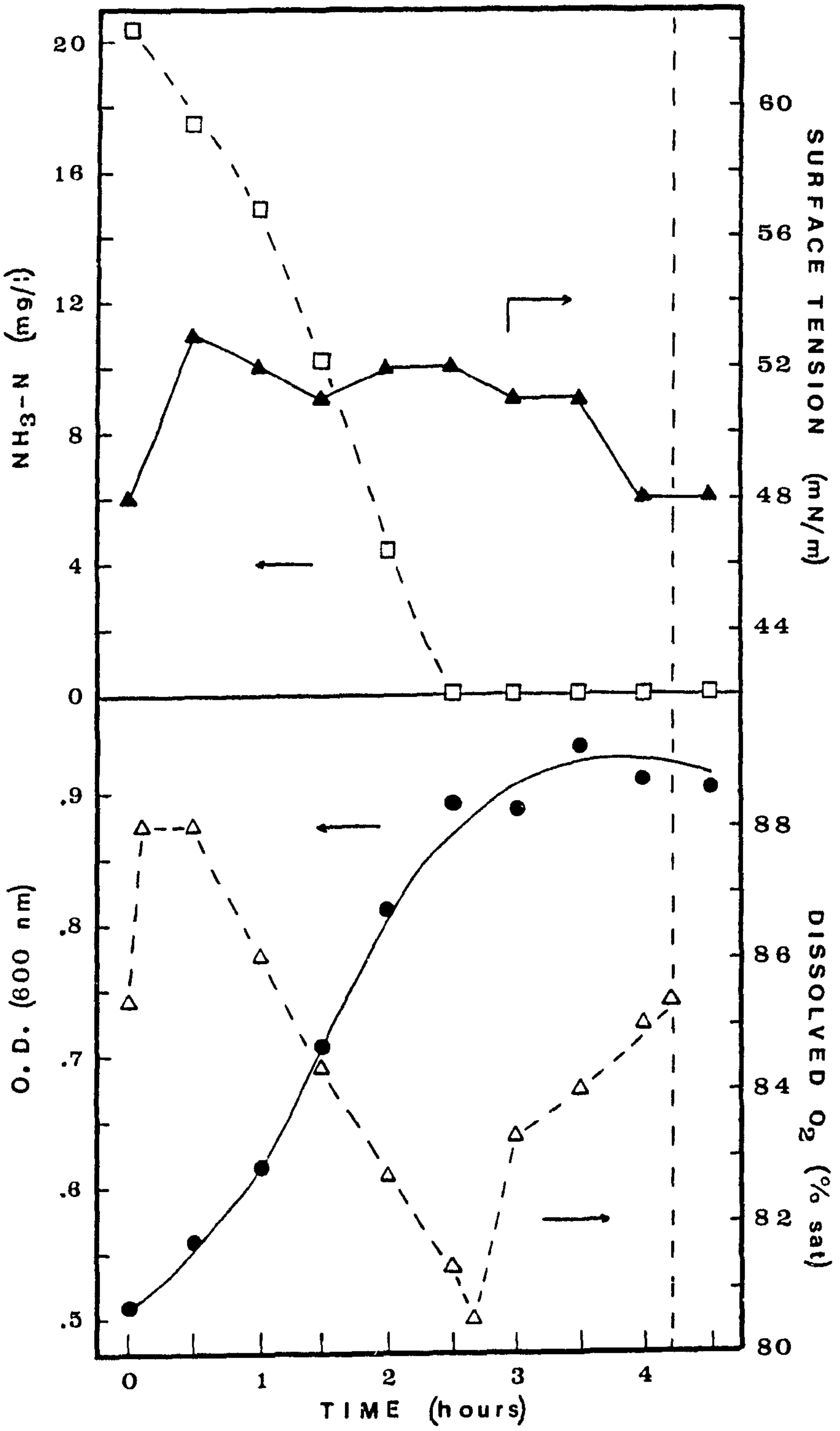

NH3-N (mg/l)
20
16
12
8
4
0
SURFACE TENSION (mN/m)
60
56
52
48
44
O. D. (600 nm)
.9
.8
.7
.6
.5
DISSOLVED O2 (% sat)
88
86
84
82
80
0
1
2
3
4
TIME (hours)

Figure 33

Nutrient Cycle Number 36 with a 60-minute Starvation Period

Shown are changes in nitrogen (□), surface tension (▲), biomass (●) and dissolved oxygen concentration (△) over the duration of a nutrient cycle with an added 60-minute starvation period.

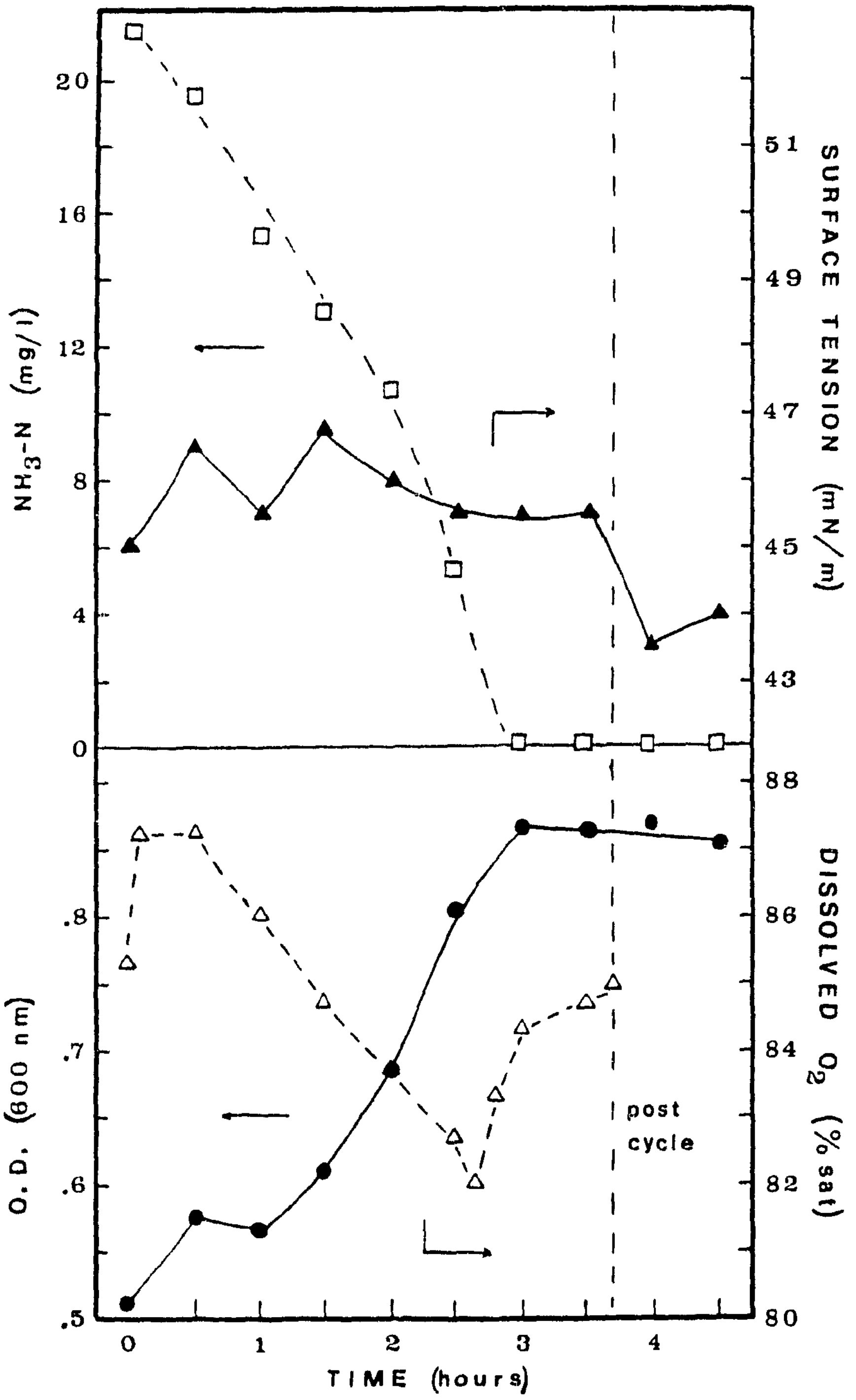
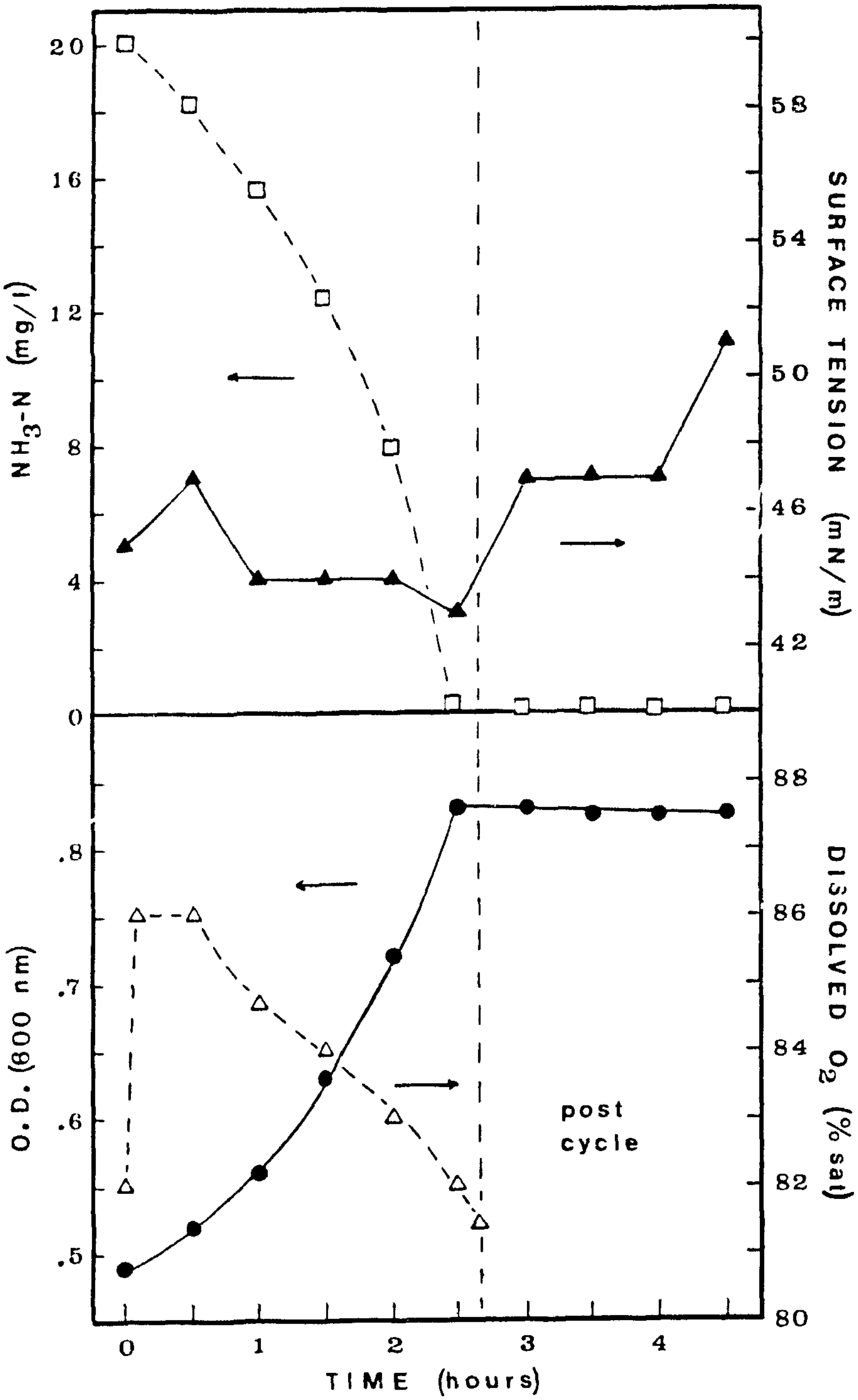

NH$_3$-N (mg/l)
20
16
12
8
4
0
SURFACE TENSION (mN/m)
51
49
47
45
43
O.D. (600 nm)
.8
.7
.6
.5
DISSOLVED O$_2$ (% sat)
88
86
84
82
80
post
cycle
0
1
2
3
4
TIME (hours)

Figure 34

Nutrient Cycle Number 66 with No Added Starvation Period

Shown are changes in nitrogen (□), surface tension (▲), biomass (●) and dissolved oxygen concentration (Δ) over the duration of a nutrient cycle with no added starvation period.


20
16
12
8
4
0
NH$_3$-N (mg/l)
58
54
50
46
42
SURFACE TENSION (mN/m)
.8
.7
.6
.5
O.D. (600 nm)
88
86
84
82
80
DISSOLVED O$_2$ (% sat)
post cycle
0
1
2
3
4
TIME (hours)

initial startup period of about ten cell generations the system was operated for another 26 nutrient cycles. At that point the control programme was restarted with a 120 minute starvation period added onto the end of each cycle. The results from the period of zero starvation correspond very closely to the previous experiment run under these same conditions. The average biomass doubling time was 150 minutes and the surface tension ranged between 30 and 34 mN/m. The addition of the 120 minutes of starvation resulted in an increase in the net doubling time of 18 minutes or 12.2%. However, the effect on surface tension was even more significant, with the average increasing to 45 mN/m. This is the value typically obtained at the end of a batch culture using this same medium.

4.3.2.3 Residual nitrogen

The experiment was performed using Version 6 of the control programme. This allowed the selection of a minimum dissolved oxygen that would be used to trigger the nutrient cycling. With an appropriate selection, the system can be operated with any desired residual nutrient concentration without losing stability. This type of operation more closely resembles a chemostat in which there is always a certain residual nutrient level that controls the growth rate.

However, in this case the limiting nutrient concentration is still being periodically varied, with the period equal to the biomass doubling time. An important aspect of continuous phasing is that the harvest and dosing volumes are both equal to one half of the reactor working volume. This ensures that each successive generation of cells is grown under an identical nutrient environment. When a change is made in the medium (as is possible with Version 6 of the programme), a new equilibrium is established within only a few cycles and is maintained for all subsequent cycles. Therefore, there is no accumulation of residual nutrient in the broth. Table 7 compares the results of four experiments with various residual concentrations of nitrogen. The minimum biomass doubling time is between 95 and 100 minutes, obtained with either 8 or 11 mg/L of nitrogen. Decreasing the residual nitrogen to 3 mg/L increases the doubling time by about 20 to 25 minutes. Operating in the normal phasing mode with no residual nitrogen increases the doubling time by an additional 20 minutes to between 140 and 150 minutes. The residual nitrogen concentration apparently had no significant effect on the biomass concentration, which averaged between 0.23 and 0.27 g/L. These results are consistent with previous observations that the time of nutrient exhaustion is the most important for control of cellular metabolism. The effects of nutrient limitation are emphasized when an additional period of starvation is imposed on the cells, and these effects are

Table 7

The Effects of Residual Nitrogen on Specific Growth Rate

Minimum D.O. (% sat.)	Residual nitrogen (mg/L)	Doubling time (min.) Max.	Average*	Min.
84	11	135	114	99
83	8	107	102	96
81	3	145	129	119
80	0	164	150	139

* average of at least 14 consecutive cycles.

always revealed by the cells during subsequent cycles.

4.3.2.4 Dissolved oxygen

In addition to providing an effective means for obtaining feedback control, the change in the dissolved oxygen concentration can also reveal at least qualitative differences in cellular metabolism. For example, under certain cultural conditions the cell population apparently respires with a regular oscillation of about 20 minutes duration, giving a trace of dissolved oxygen as shown in Figure 35. This oscillation can be very stable, repeating itself 4 or 5 times each nutrient cycle. Changing the cultural conditions can eliminate the oscillation, however, reverting back to the original conditions will usually initiate it again. This is illustrated in Figure 35 where the addition of a 120 minute starvation period completely damped the oscillation within two nutrient cycles, and within two cycles of eliminating the starvation period, the oscillation had returned. The same phenomenon was observed while testing the effects of manganese (Figure 36). Increasing the manganese in the medium from 1 x 10^{-5} to 1 x 10^{-4} molar damped the oscillation within two nutrient cycles. A subsequent decrease in manganese to the former level renewed the oscillation within three nutrient cycles. This was repeated several times with the same result.

Figure 35

Regular Oscillations in the Dissolved Oxygen Concentration Before and After Addition of a Starvation Period

Arrows indicate the times at which fresh nutrients were added.

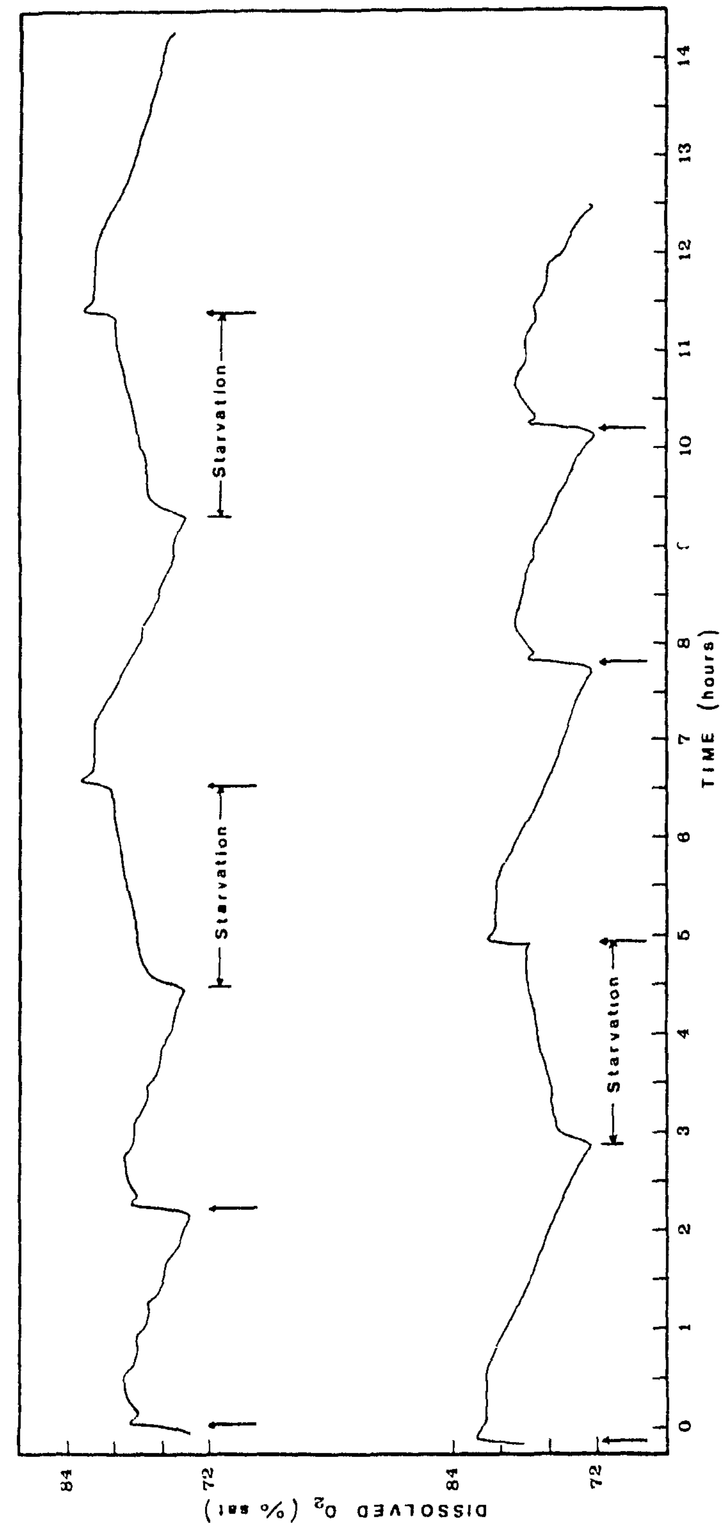
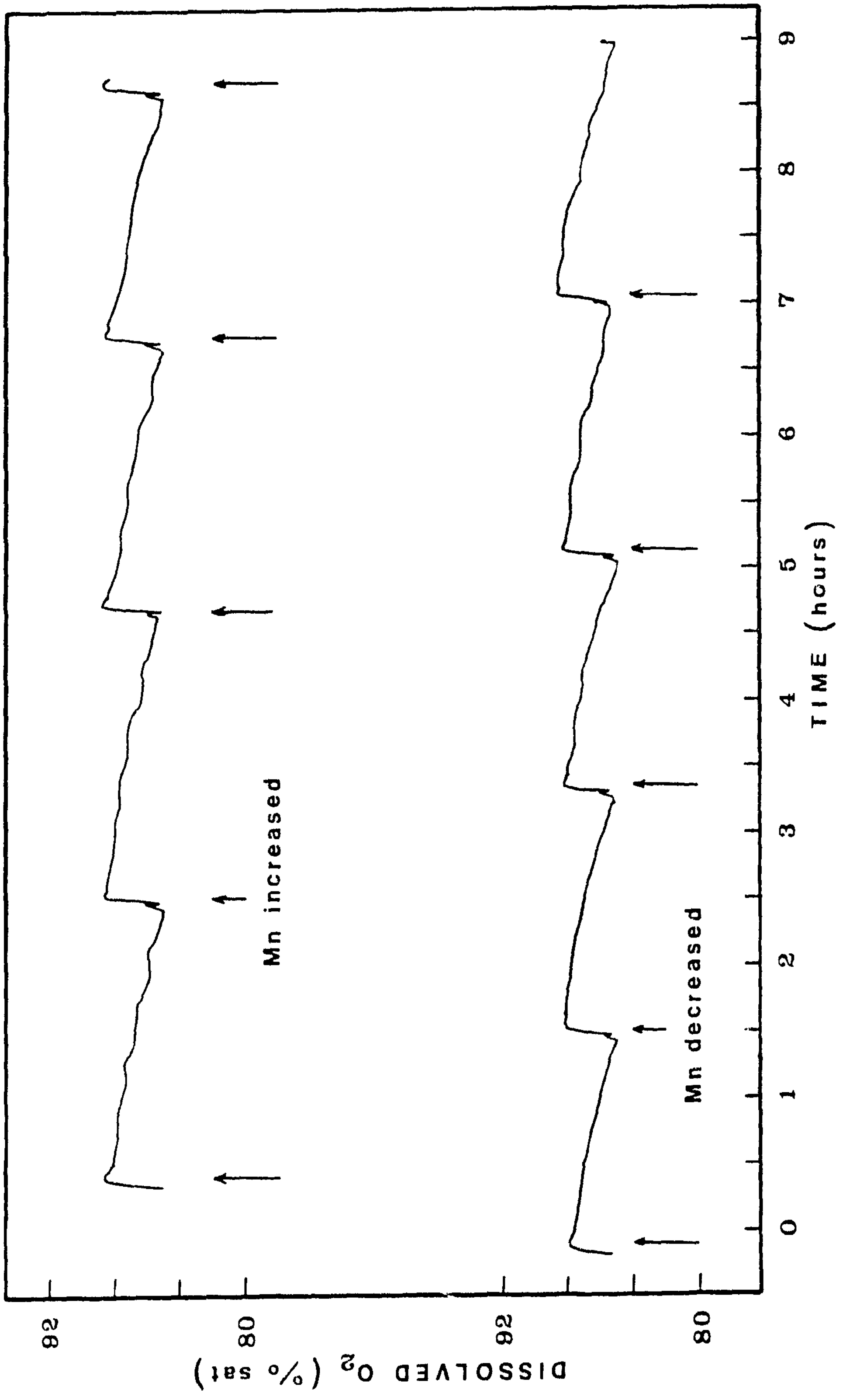

84
72
84
72
DISSOLVED O_2 (% sat)
Starvation
Starvation
Starvation
0
1
2
3
4
5
6
7
8
10
11
12
13
14
TIME (hours)

Figure 36

Regular Oscillations in the Dissolved Oxygen Concentration Before and After Increasing the Manganese Concentration

Arrows indicate the times at which fresh nutrients were added.


Mn increased
Mn decreased
TIME (hours)
0
1
2
3
4
5
6
7
8
9
DISSOLVED O_2 (% sat)
80
86
80
86

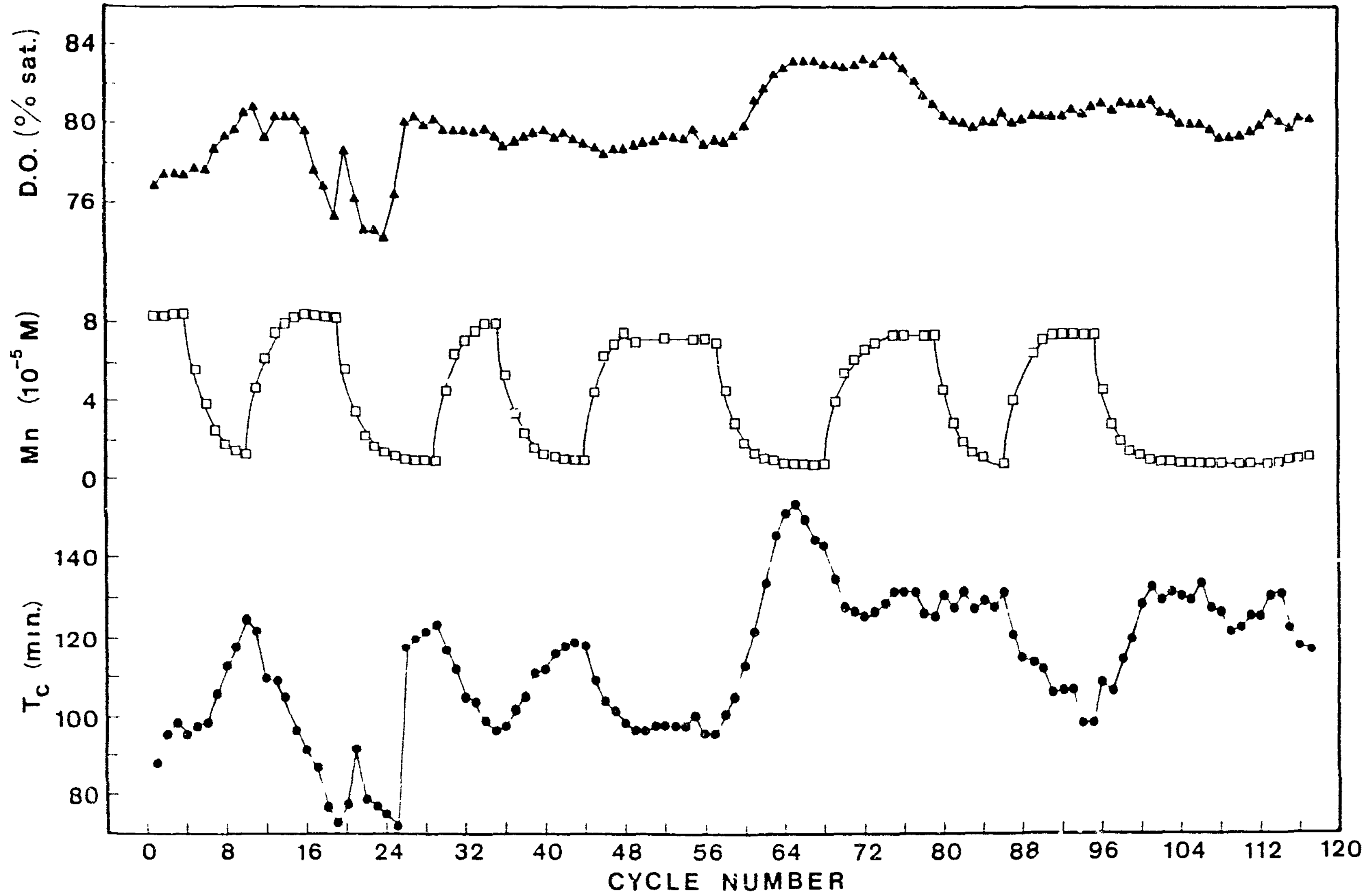
4.3.2.5. Dynamic response

Two experiments were conducted to characterize the dynamic response of the cell population to changes in the manganese concentration in the broth. The iron concentration was decreased to 3.3 x 10^{-5} molar, while all other nutrients were the same as the standard nitrogen limited medium (Table 1). The results of these experiments are illustrated in Figures 37 and 38. The manganese concentration was varied to approximate a square wave by periodically switching between two media, one containing manganese at 1 x 10^{4} molar and the other at 1 x 10^{-5} molar. The number of successive cell generations that were subjected to each concentration was not constant and varied between 6 and 22 in the first experiment (Figure 37), with six complete square wave cycles. The second experiment (Figure 38) employed only 2.5 manganese cycles, however, they were of longer duration. Also, in the second experiment a single 7-hour starvation period was added to the end of cycle 65. The results indicate that for the range of manganese concentrations tested, the biomass doubling time (as reflected by the cycle time) is a function of the manganese concentration. However, the change in specific growth rate is not immediate and requires several generations of cells before a consistent doubling time is attained. Based on the results of the second experiment, it seems that after changing the manganese concentration, it may require at least 12

Figure 37

Response of the Cell Population to Step Changes in the Manganese Concentration of Short Duration

Shown are changes in the dissolved oxygen concentration at the end of each cycle (▲) and the cycle time (●) as they relate to the residual manganese concentration in the broth (□).

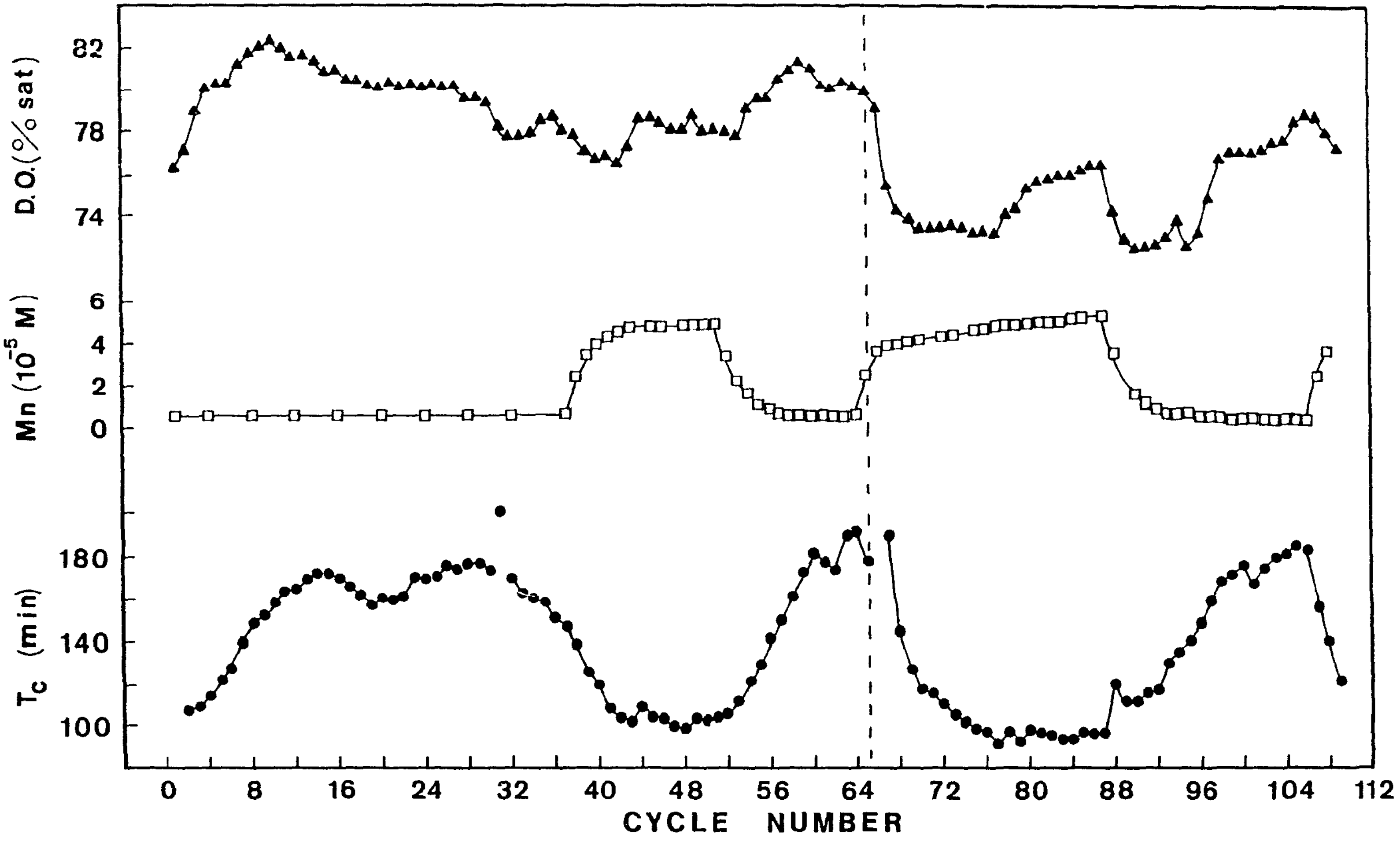


D.O. (% sat.)
84
80
76
Mn (10^{-5} M)
8
4
0
T_c (min.)
140
120
100
80
0
8
16
24
32
40
48
56
64
72
80
88
96
104
112
120
CYCLE NUMBER

Figure 38

Response of the Cell Population to Step Changes in the Manganese Concentration of Long Duration

Shown are changes in the dissolved oxygen concentration at the end of each cycle (▲) and the nutrient cycle time (●) as they relate to the residual manganese concentration in the broth (□). A 7 hour starvation period was added to the end of cycle 65.


D.O.(% sat)
82
78
74
Mn (10^{-5} M)
6
4
2
0
T_c (min)
180
140
100
0
8
16
24
32
40
48
56
64
72
80
88
96
104
112
CYCLE NUMBER

generations of cells before achieving a constant specific growth rate, especially if the level of manganese has been reduced. These non-linear dynamics can be conveniently illustrated by plotting the cycle time as a function of the manganese concentration and connecting the points by their temporal association. This was done for the data from the second experiment and is presented in Figure 39. Each path can be called a trajectory and the whole figure is a phase plane portrait of the dynamic behaviour (Glass and Mackey 1988). It would appear that as the number of successive generations of cells subjected to a given manganese concentration becomes large, the relationship between specific growth rate and manganese concentration would likely converge to a straight line, within the limits of 190 minutes at 0.6 x 10^{-5} molar and 93 minutes at 5.2 x 10^{-5} molar.

The doubling time can, therefore, be represented by the following equation:

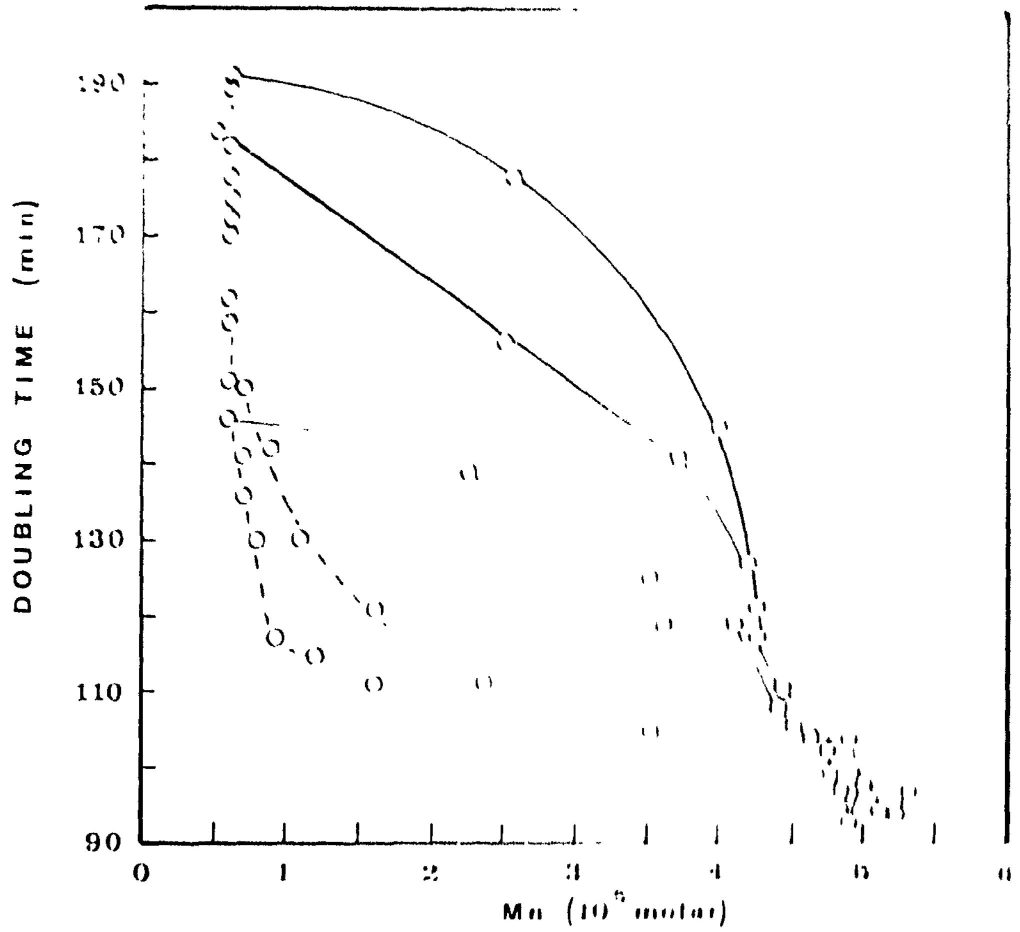
$$T_d = 203 - ((Mn) \cdot 21.7) \qquad (6)$$

where Mn represents the concentration of manganese in units of 10^{-5} molar and T_d is in minutes. It is important to note that any single generation of cells may have a doubling time at any value between about 90 and 200 minutes, regardless of the concentration of manganese in the medium. This equation

Figure 39

The Phase Plane Portrait of the
Dynamic Response to Manganese

Shown is the change in biomass doubling time, reflected by the nutrient cycle time, as the residual manganese concentration is changed. Note that the relationship between specific growth rate and manganese requires interpretation over many successive cell generations. The dashed lines represent trajectories with decreasing concentrations of manganese and the solid lines increasing concentrations.



DOUBLING TIME (min)
190
170
150
130
110
90
0
1
2
3
4
5
6
Mn (10^5 molar)

does not account for the dynamic response of the population and is only valid when the manganese concentration is constant for many successive generations.

4.3.5 Discussion

The conventional method of continuous phasing which imposes an arbitrary period of nutrient cycling on the cell population, revealed three important phenomena:

i) the interaction between the period of nutrient cycling and the growth rate of the cells;

ii) the changing metabolic response of the population to successive nutrient cycles;

iii) the importance of the time and duration of nutrient starvation in affecting cellular metabolism.

The interaction between the imposed period of nutrient cycling and the metabolism of the cells precluded the possibility of systematically studying the other two phenomena. As the metabolic response of the cells changed, the relationship between the cellular metabolism and the imposed nutrient cycle also changed, thus making it impossible to characterize the metabolism based on a constant relationship between the cell and the environment. This was particularly evident during the growth of B. subtilis ATCC 21332, where the surfactant production appeared to be strongly influenced by the period of nutrient starvation. The changing specific growth rate, in response to a particular period of nutrient cycling, resulted in a continuously changing period of starvation. This dilemma was overcome by the development of the feedback system to control the period of nutrient cycling based on the metabolic requirements of each generation of cells. Identification of the precise time of nutrient exhaustion, by monitoring the respiration rate, allows the periodic nature of the environment to be imposed by the metabolic changes in the cell population. Thus, each successive generation of cells is subjected to an environment that has both the same nutrient composition and a constant temporal relationship to the metabolism. This is ensured by the combination of the feedback control and the removal and replenishment of precisely one half of the broth each cycle.

The results of the experiments utilizing feedback control illustrate the power of the technique for increasing the scope of possible experimentation and potential for achieving significantly higher production rates. The first experiment, utilizing a minimum period of nutrient starvation, demonstrated that high levels of surfactant production could be maintained for at least 80 consecutive generations of cells with controlled stability, even after changes to the medium and a power failure. Subsequent experiments were able to further investigate the effects of nutrient starvation by programming the control system to automatically adjust the period of nutrient cycling, to ensure that each successive generation of cells was subjected to the same starvation period. Another control programme was used to operate the system with various concentrations of residual nitrogen, thus completely eliminating starvation.

These experiments confirmed the importance of the period of nutrient starvation in controlling cellular metabolism. Although it appears that a nutrient limitation is required for inducing surfactant production, the severity of the starvation determines the rapidity of the subsequent repression. Thus, maximum productivity was obtained with "minimal" starvation. Even though each successive generation of cells was subjected to a nutrient limitation, the period of nutrient starvation was minimized with the feedback control system. Introducing an extended starvation period accelerated the decrease in surfactant production over succeeding generations. The effects of starvation were also seen on the specific growth rate. Maintaining some residual nitrogen in the broth resulted in significantly faster biomass doubling times, decreasing from about 150 minutes with zero residual nitrogen to 100 minutes with 8 mg/L residual nitrogen. Therefore, the technique could also be used to evaluate kinetic constants analogous to chemostat operation. However, in this case the nutrient concentrations would be related to the dynamics of the cell cycle and not just to an average specific growth rate.

The results are consistent with the theory that surfactin is produced in response to an environmental stress, resulting in significant metabolic changes in the cells. Stress responses are apparently ubiquitous among living cells and have been documented in bacteria, yeast, insects, plants and mammalian cells (Goochie and Passini 1988). In bacteria the stress response has been studied mostly in E. coli, especially using heat shock (Neidhardt and Van Bogelen 1987), but also as a result of damage to DNA (Walker 1987) and amino acid deficiency (Cashel and Rudd 1987). A general feature of cellular metabolism appears to be the effect of the environment as a modulator of metabolic rate if the stimulus (such as temperature or nutrient concentration) is within a certain "normal" range for the organism. When the stimulus is outside of this range then the metabolism undergoes a much more severe reaction, resulting in the use of completely different enzymatic systems (Neidhardt and Van Bogelen 1987). For example, when amino acid deficiency is induced in E. coli creating the so-called stringent response, the production of many proteins are inhibited but almost equal numbers are stimulated into production (Cashel and Rudd 1987).

Changes to the supply of an essential nutrient, such as nitrogen, can also result in large metabolic adaptation. In E. coli decreasing the ammonia concentration below 1 x 10^{3} molar results in the activation of operons that encode products to facilitate the assimilation of low concentrations of ammonia and then the utilization of alternative sources of nitrogen (Magasanik and Neidhardt 1987). Continuous phasing results in exhaustion of an essential nutrient every generation of cells. Thus, unless the cellular metabolism became organized in a manner that was anticipating a nutrient limitation, each generation of cells would be expected to exhibit a rather severe metabolic reaction. Surfactin is a lipopeptide that has been shown to be membrane active (Bernheimer and Avigad 1970) and is very structurally similar to the iturins that produce ion-selective channels in black lipid membranes (Maget-Dana et al. 1985a, 1985b). Therefore, it is possible that part of the stress response to the nutrient limitation is a change in the membrane permeability as a result of the over-production of surfactin. However, this over-production of a complex metabolite, such as surfactin, would require considerable energy and could not be maintained as the period of nutrient starvation proceeded. Subsequent replenishment of the limiting nutrient would allow the cells to recover but, presumably, there would be residual effects on the metabolism of the next generation. These effects are apparently proportional to the duration of the starvation period and are also cummulative over successive generations of cells. The net effect is eventually a marked decrease in surfactin production, as the population of cells becomes conditioned to the nutrient limitation and the stress response is virtually eliminated.

The apparent metabolic changes occurring in the population may be a result of a shift in the distribution of individuals within the population, each with slightly different capabilities. Since there is a metabolic advantage to saving energy by minimizing the stress response, the technique of continuous phasing may select for the individuals that express the minimum stress response to the nutrient limitation. This possibility was examined by conducting two batch cultures, one inoculated with cells that were obtained directly from a phased culture that was no longer producing surfactin, and the other batch inoculated with cells that had also come from the same phased culture but had been first plated on nutrient agar for 24 hours and then grown in a shake flask for another 36 hours. So, both inocula originated from the same source, however, the second inoculum was allowed to grow for 10 to 20 generations without a nutrient limitation prior to being grown as a conventional batch culture. The results from these two batches are illustrated in Figures 40 and 41. There are only slight differences in the growth kinetics, both attaining a minimum doubling time of about 1.7 hours which is characteristic of this medium. However, Figure 41 shows how surfactin production remained poor in the first batch but was restored to the typical pattern, shown by B. subtilis ATCC 21332, in the second batch. The surface tension dropped rapidly, 10 to 15 hours after inoculation, to less than 30 mN/m. The results, therefore, indicate that no

Figure 40

Change in Biomass Concentration During Two Batch Cultures Inoculated from a Phased Culture

The inocula for both batches were obtained from a culture subjected to nutrient cycling for 108 generations, however, the inoculum for the batch represented by the closed symbols (●) was first plated and then grown in a shake flask. The other batch (o) was inoculated directly from the phased culture.

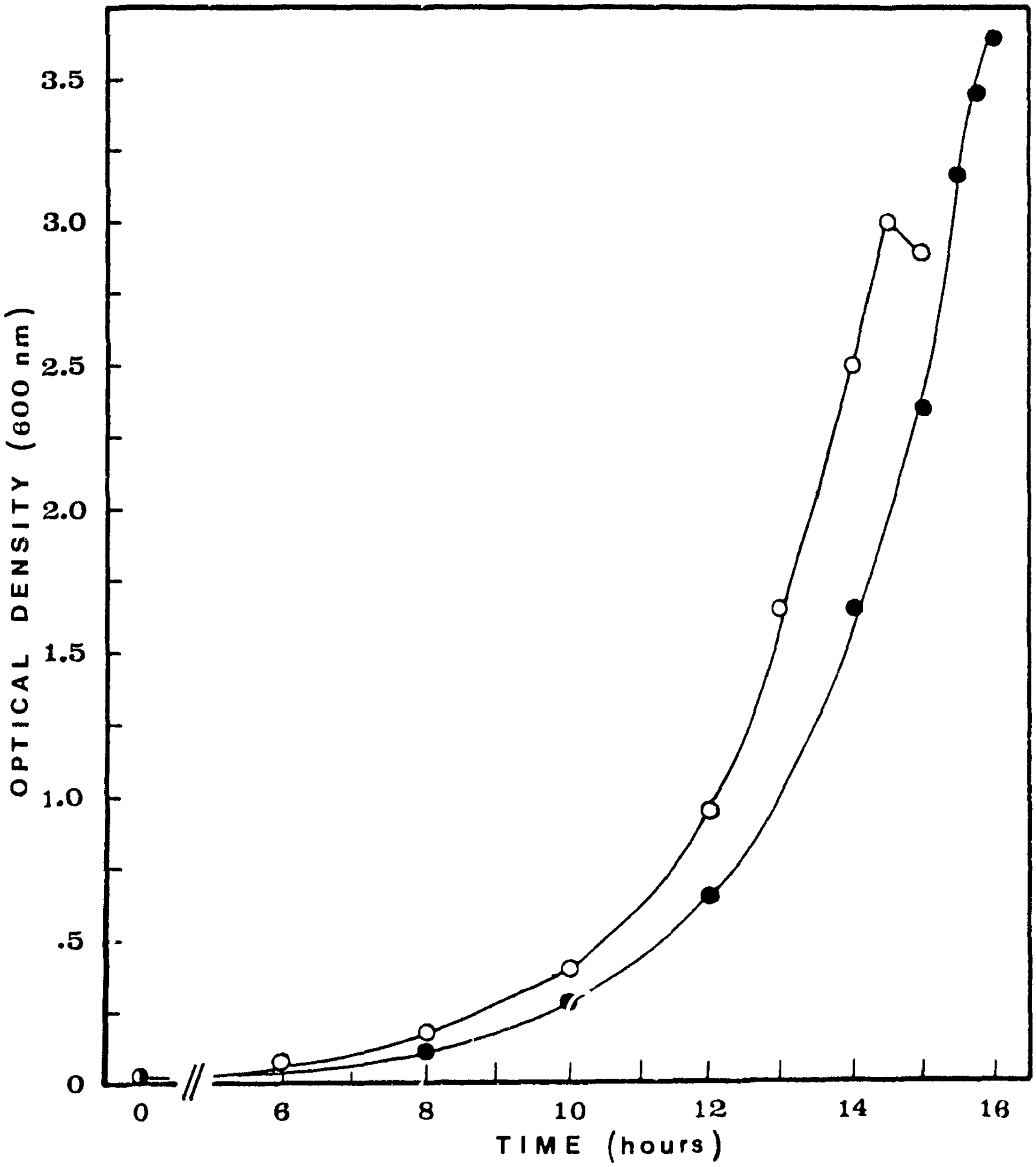
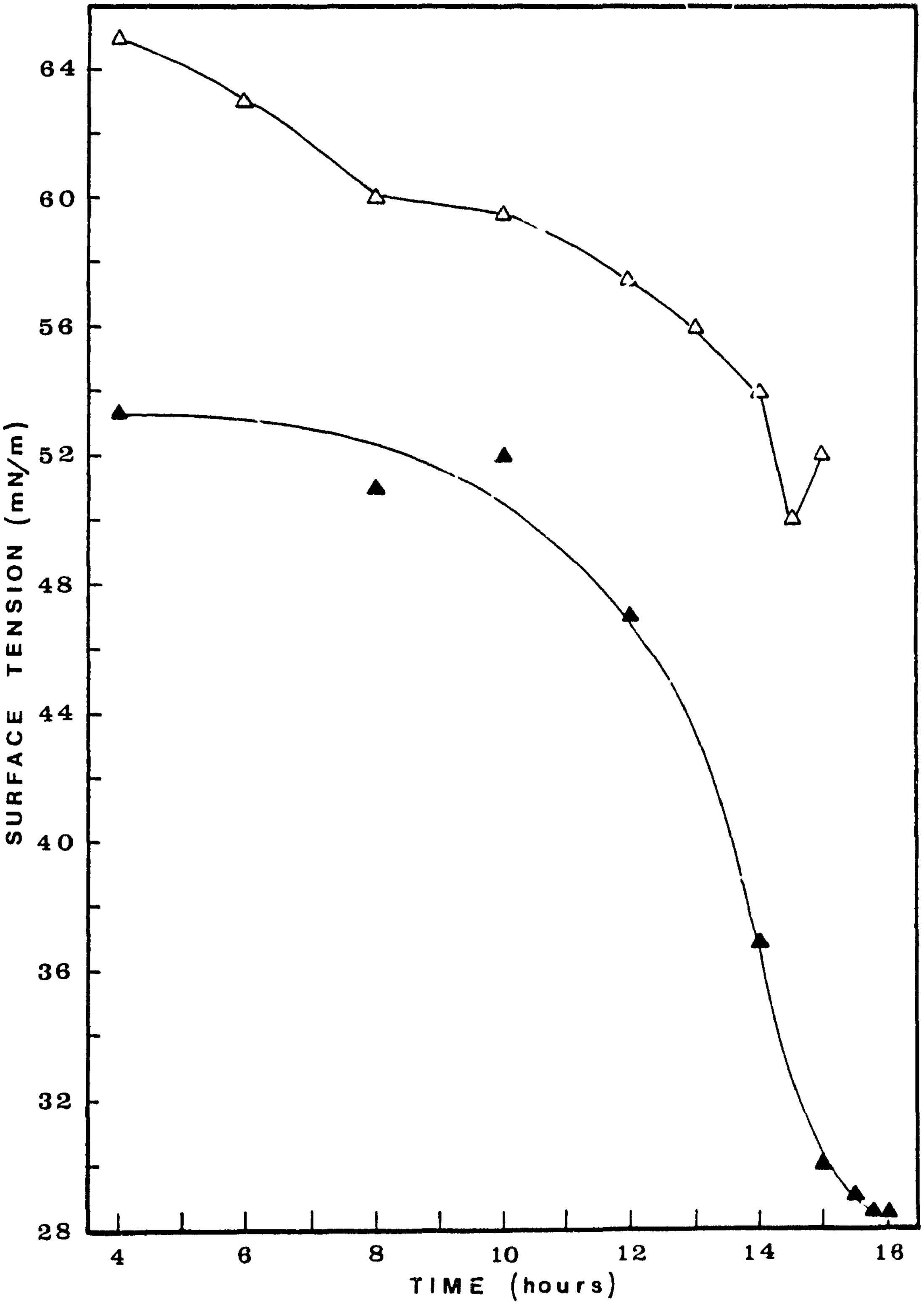

OPTICAL DENSITY (600 nm)
3.5
3.0
2.5
2.0
1.5
1.0
.5
0
0
6
8
10
12
14
16
TIME (hours)

Figure 41

Change in Surface Tension During Two Batch Cultures Inoculated from a Phased Culture

The inocula for both batches were obtained from a culture subjected to nutrient cycling for 108 generations, however, the inoculum for the batch represented by the closed symbols (▲) was first plated and then grown in a shake flask. The other batch (△) was inoculated directly from the phased culture.


SURFACE TENSION (mN/m)
64
60
56
52
48
44
40
36
32
28
TIME (hours)
4
6
8
10
12
14
16

irreversible genetic mutation had occurred during continuous phasing. The population of cells retained the capability for surfactin production. The metabolic condition that had resulted from an extended period of nutrient cycling (108 generations) reverted back to an "unconditioned" state while growing on the nutrient agar and in the shake flask. If the continuous phasing had resulted in the domination of the population by a "mutant", then it is highly improbable that the population would have spontaneously mutated back to once again give the pattern typical of surfactin production in batch culture. This implies that the genetic integrity of the population is maintained even if apparent metabolic differences have been selected for. Subsequent changes to the environment can return the population to its previous state.

One of the main advantages to the addition of feedback control to continuous phasing is the inherent stability of the system. If the composition of the medium or any other environmental factor that affects specific growth rate is changed, the control system automatically adapts the period of nutrient cycling to reflect these changes. This provides a convenient method for studying the dynamic response of a culture to changes in the environment without the danger of cell wash-out. The dynamic response of B. subtilis ATCC 21332 to step changes in the concentration of manganese revealed that growth of up to 12 generations of cells was required at the same manganese concentration before a consistent doubling time was attained. Eventually the specific growth rate approached a value that appeared to be linearly correlated with the manganese concentration between 1 x 10^{-5} and 1 x 10^{-4} molar. When a dynamic system is perturbed, for example by a step change in the environment, and then returns to its original state when the environment is restored, it is said to be a stable limit cycle (Glass and Mackey 1988). Continuous phasing with feedback control allowed the dynamic response of the cell population to be represented by a phase plan portrait that describes cell growth as a stable limit cycle. This means that the specific growth rate is a predictable function of the manganese concentration in the medium. However, the dynamic nature of the system requires that any model account for the metabolic history of previous generations. Without this information, it is not possible to predict the response of any single generation. Only the final specific growth rate that will be approached can be known, and only if the same nutrient environment is present for each generation, and for a sufficient number of generations. That is, the specific growth rate, or doubling time as shown in Figure 39, will eventually approach a limit as defined by the composition of the nutrient medium (for example equation (6)). However, the growth of any single generation of cells may be far from the limit, with the growth of subsequent generations approaching on one of any number of tangents. This phenomenon is likely the explanation for the inherent variability associated with the lag period in conventional batch cultures. The time required to approach the logarithmic growth rate in any specific medium will be highly dependent on the position of the inoculum on the appropriate phase plane portrait.

4.4 Product Identification

4.4.1 Procedure

The extracellular products produced during the growth of B. subtilis ATCC 21332 were recovered from the broth as described in Section 2.3. The procedure involved the acidification of the cell free broth, followed by extraction of the precipitate by chloroform. The final product(s) were further characterized by thin layer chromatography (Section 2.6) and amino acid analysis (Section 2.8). The TLC tests were performed to indicate qualitative differences in product distribution for various cultural conditions. The amino acid analyses were more quantitative and could be used to confirm the purity of the product.

4.4.2 Results

Table 8 presents the data from the TLC of products obtained after batch growth in the original medium of Cooper et al. (1981). Six different spots were identified on the plate, with the top three being the most abundant based on

Table 8

TLC of Products from Batch Growth in the Medium of Cooper et al. (1981)

Relative Quantity[1]	R_f[2]
1	0.80-0.82
3	0.50-0.67
2	0.53-0.56
4	0.39-0.44
5	0.26-0.32
6	0.16

1. Determined by visual examination after development in iodine vapours.
2. Chloroform/methanol/water solvent mixture of 65/25/4 by volume.

Table 9

TLC of Products from Phased Growth in Nitrogen-limited Medium

Relative Quantity[1]	R_f[2]	R_f[3]	R_f[4]
1	0.80	0.74	0.91
2	0.67	0.79	0.91
3	0.54	0.54	0.67
4	0.32	0.32	0.53

1. Determined by visual examination after development in iodine vapours.
2. Chloroform/methanol/water solvent mixture of 65/25/4 by volume.
3. Chloroform/methanol/28% NH_4OH solvent mixture of 65/25/4 by volume.
4. Chloroform/methanol/acetic acid solvent mixture of 65/25/4 by volume.

spot intensity and size. This is similar to the product distribution normally obtained from a phased culture, shown in Table 9. The same three spots with the high R_f values were observed. TLC with an acidic solvent indicated that the products became more hydrophobic, presumably as a result of the protonation of the charged amino acids, glutamic and aspartic. In the basic solvent there was no change for the lower two R_f values. However, the upper spot showed a slight reduction in solvent mobility (or hydrophobicity) and the second highest spot increased in mobility.

The results from the experiment with controlled starvation (see Section 4.3.2.2) are presented in Table 10. Apparently the product distributions were slightly affected by the starvation period, however, in all cases the spot with the highest R_f value dominated. Relative amounts of the other products changed only slightly, except in the case of zero starvation. Under this condition, the spot with an R_f value between 0.36 to 0.38 went from very faintly visible to a dark concentrated spot.

The results of the amino acid analyses, presented in Table 11, are consistent with the expectation that surfactin is the principle lipopeptide being produced. The crude acid precipitate contains a considerable fraction of material other than surfactin. Based on the analysis of 2.5 μg of product, 100% surfactin would result in a total of 16.9 nmoles of amino acids, composed of leucine, valine, aspartic and glutamic

Table 10

TLC of Products from Phased Growth with Various Starvation Periods

Starvation period (min)	R_f values[1]					
120	0.78	0.67	0.58	-	-	0.26
90	0.81	0.62	-	0.47	0.38	0.28
60	0.81	-	0.56	0.45	0.36	0.28
30	0.82	-	0.54	0.45	0.36	0.25
0	0.82	-	0.59	-	0.37	-

[1] Chloroform/methanol/water solvent mixture of 65/25/4 by volume.

Table 11

The Results of the Amino Acid Analysis of Various Product Fractions

Fraction	Amino Acids (nmoles in 2.5 μg)						
	Leu	Asp	Glu	Val	Total[1]	Ratio[2]	Purity(%)[3]
Acid precipitate	2.69	0.83	0.86	0.91	6.27	4/1.2/1.3/1.4	31
Chloroform extract	7.31	2.02	2.07	2.38	14.45	4/1.1/1.1/1.3	82
Residue	0.35	0.23	0.26	0.15	2.09	4/2.6/3.0/1.7	6
TLC fraction R_f = 0.65-0.86	1.22	0.34	0.35	0.41	2.79	4/1.1/1.1/1.3	NA
TLC fraction R_f = 0.44-0.65	1.26	0.34	0.35	0.39	2.76	4/1.1/1.1/1.2	NA
TLC fraction R_f = 0.23-0.44	27.55	7.46	7.62	9.10	53.75	4/1.1/1.1/1.3	NA

1. Represents the total nmoles of all amino acids in the 2.5 μg of sample, including free ammonia

2. Ratio of Leu/Asp/Glu/Val is ideally 4/1/1/1 for surfactin

3. 100% purity is based on a total of 16.9 nmoles of amino acids in 2.5 μg of surfactin.

acids. The acid precipitate contains only 5.29 nmoles of these four, and a total of 6.27 nmoles of amino acids. This indicates that the main contaminant is not a protein. Subsequent chloroform extraction of the precipitate increases the purity to over 80%, ruling out a lipid as the contaminant. Therefore, it would seem that the surfactin molecule is associated with some hydrophilic compound such as a carbohydrate.

The chloroform extract was further separated by TLC in a chloroform/methanol/water solvent mixture (65/25/4 by volume). Three fractions were collected and analyzed for amino acids. There was insufficient sample to calculate purity based on the total material, as was possible for the other samples, however, based on total amino acids the samples averaged 98% purity. Therefore, it is apparent that all three major fractions separated by TLC are surfactin. The different solvent mobilities are possibly a result of different conformations of the hydrophilic ring of amino acids.

4.4.3 Discussion

Previous TLC work by Vater (1986) found that surfactin had an R_f value of 0.65 in a solvent mixture of chloroform/methanol/water (65/25/4 by volume). The same value was obtained in this study, however, several other product fractions were also obtained based on TLC. The distribution of the various fractions seems to have been affected in some cases by the cultural conditions used for production. The method for purification of the products is designed to recover only lipopeptides, although it is possible that other compounds may be intimately associated with the surfactin and survive the purification procedure. The amino acid analyses have shown that about 98% of the proteinaceous material in the purified product belongs to surfactin. However, there is about 20% of the product that is not surfactin, unless, the fatty acid chain is actually longer than is reported by Kakinuma et al. (1969a, 1969b). No analysis of the fatty acids was performed in this study. The amino acid analyses of separate TLC fractions also show that surfactin can have different R_f values in the same solvent. The cause of this is unknown. It is possible that surfactin is produced in different physical conformations or that these conformations are an artifact from the extraction and purification procedure. In either case, TLC must be considered as an unreliable method for product identification.

4.5 Oxygen Transfer Coefficient

4.5.1 Procedure

The volumetric oxygen transfer coefficient, represented by K_La, is a measurement of the overall resistance to oxygen transfer from the gas to liquid phases.

$$QO_2 = K_La \ (C^* - C) \qquad (7)$$

The flux of oxygen, QO_2 in moles per liter-hour, is the product of the K_La (per hour) and the driving force $C^* - C$, the difference in oxygen concentration in moles per liter at the gas-liquid interface, C^* and in the bulk liquid, C. The K_La is composed of two terms, K_L the inverse of the film resistance and 'a' the gas-liquid interfacial area. Unless these terms are measured independently, it is not possible to know the relative importance of each in affecting QO_2.

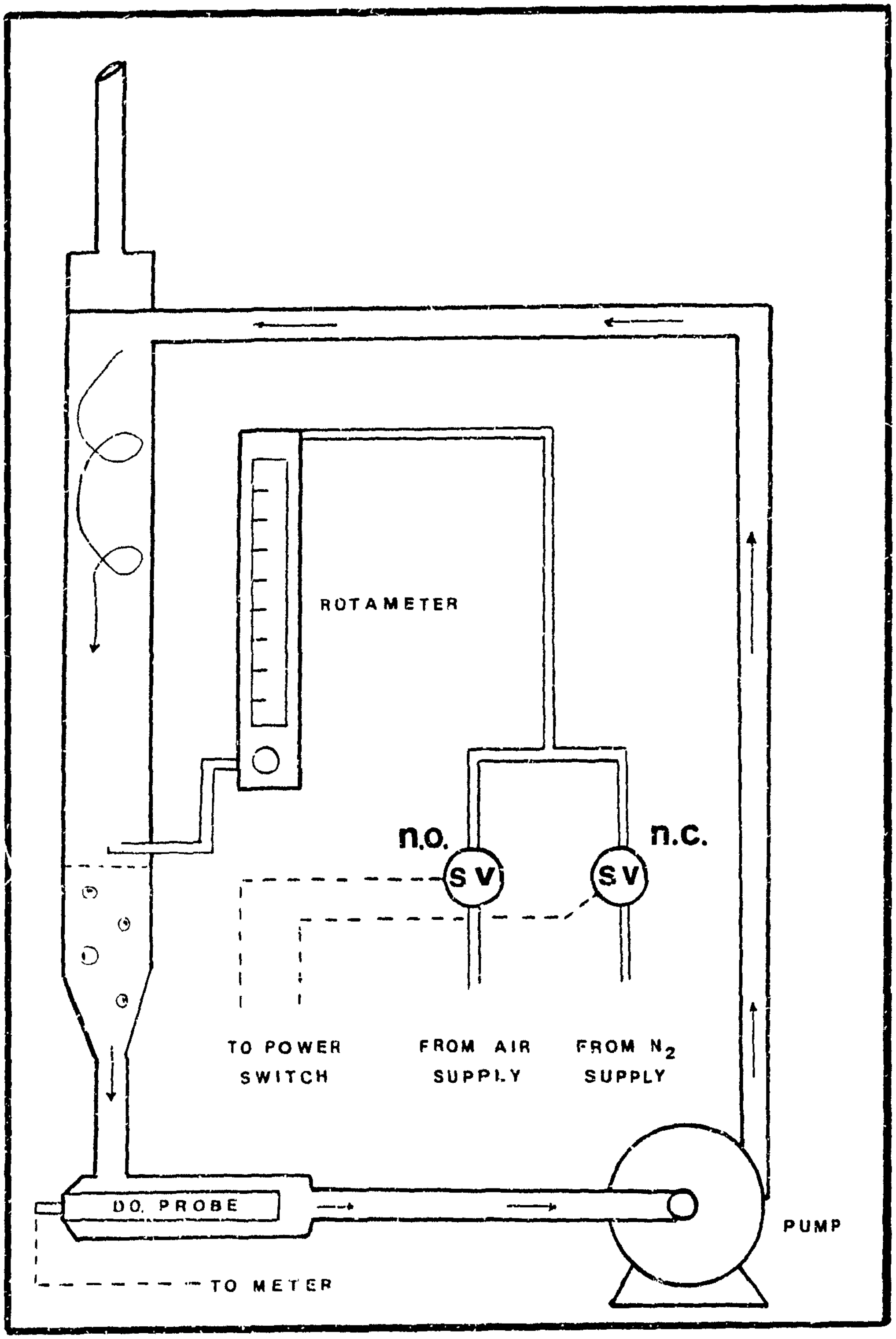
Most of the K_La measurements were made using a "gassing-in, gassing-out" technique as described by Benedek and Heideger (1971) with some modification due to the physical constraints of the cyclone column. The accuracy of this method, without compensating for the response of the probe, was verified with the sulphite oxidation method (Cooper et al. 1944). The position of the probe ensures that the effects of entrained bubbles are minimal. The liquid at this point in the recirculation loop is travelling at high velocity (46 cm/s), the resulting turbulence ensuring that entrained bubbles cannot adhere to the membrane of the probe.

For the "gassing-in, gassing-out" experiments the cyclone column was set up as illustrated in Figure 42. Two electrically actuated solenoid valves were used for control of the gas flows, nitrogen and air. The operating procedure

Figure 42

Apparatus for Determining the Volumetric Oxygen Transfer Coefficient

Shown are the solenoid valves (SV) used for control of the nitrogen and air during the gassing-in and gassing-out technique. The air supply is normally open (n.o.) and nitrogen normally closed (n.c.).


ROTAMETER
n.o.
SV
n.c.
SV
TO POWER SWITCH
FROM AIR SUPPLY
FROM N2 SUPPLY
DO. PROBE
PUMP
TO METER

was as follows:

i) The cyclone column was filled with the desired volume of liquid and the recirculating pump was started.

ii) The two solenoid valves were energized, thereby opening the flow of nitrogen and closing the flow of air. With the gas flow rate set to 1.0 L/min this condition was maintained until the dissolved oxygen concentration in the liquid reached 0% saturation.

iii) The recirculating pump was turned off and the solenoid valves de-energized, switching the gas flow from nitrogen to air. The pump remained off for five minutes. This was necessary to ensure that the head space above the liquid in the column was completely exchanged with air.

iv) After five minutes the recirculating pump and chart recorder were switched on and the concentration of dissolved oxygen was recorded until reaching about 90% saturation.

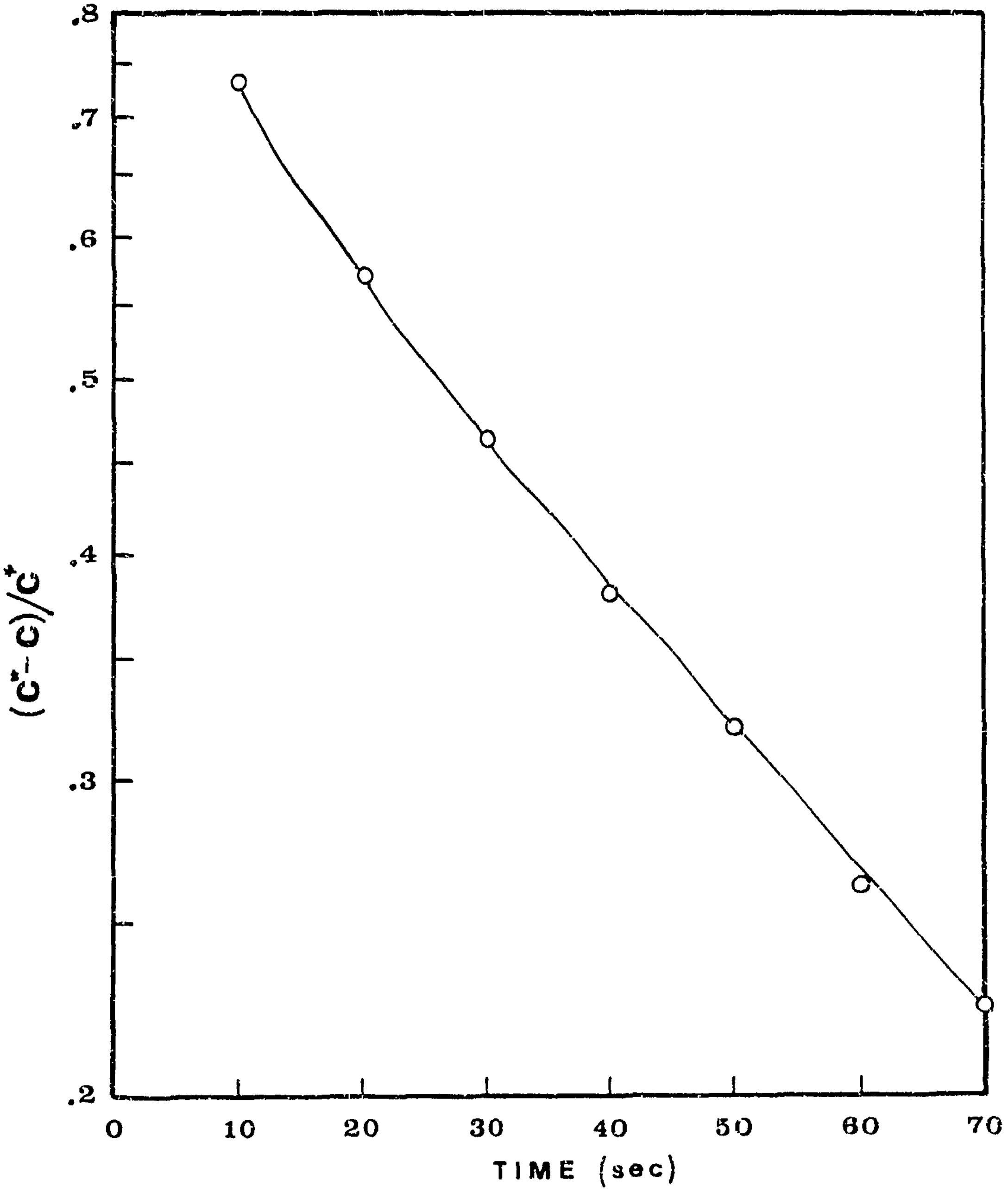
This procedure was repeated with various changes to the liquid in the reactor with respect to composition and volume. The gas flow rate and temperature were constant in all experiments at one liter per minute and 26°C respectively. The volume of the gas head space is sufficiently large to ensure a saturated value of C* over the course of a single experiment even with very low gas flow rates.

Since QO_2 is equivalent to dC/dt:

Figure 43

Change in Dissolved Oxygen Concentration while Gassing-out with Nitrogen

The slope of the line is equal to the volumetric oxygen transfer coefficient ($-K_La$). The working liquid volume is 1.75 liters of distilled water at 25°C.


.8
.7
.6
.5
.4
.3
.2
$(C^* - C)/C^*$
0
10
20
30
40
50
60
70
TIME (sec)

$$dC/dt = K_La\ (C^* - C) \qquad (8)$$

and by integration:

$$\ln [(C^* - C)/C^* - C_o)] = -K_La\ t \qquad (9)$$

where C* is the interfacial concentration, C is the measured concentration at time 't' and C_o is the initial concentration at time zero. If C_o equals zero, then plotting ln [(C*-C)/C*] versus time should yield a straight line with slope equal to K_La. This was found to be true for these experiments if the initial few seconds were ignored. A typical set of data is shown in Figure 43. The initial non-linearity could have been due to a lag before the hydrodynamic conditions stabilized or a non-linear response of the probe. It was not necessary to compensate for the probe response due to the relatively low values of K_La encountered compared to the speed of the probe's response. This assumption was substantiated by determining the volumetric oxygen transfer coefficient using the sulphite oxidation method, originally used by Cooper et al. (1944) and described in Appendix B.

The results of the sulphite oxidations indicated that there was negligible error using the dissolved oxygen probe with no correction for response. A value for K_La of 137 per hour was obtained with a 1 liter volume and 1 liter per minute of aeration compared to 140 per hour with the probe. Reducing the airflow to 0.17 liters per minute decreased the K_La to 115 per hour. However, this must be an artifact as a result of reducing C* due to inadequate air supply in the head space, since there was no change in the hydrodynamics (gas holdup or film height) with reduced aeration.

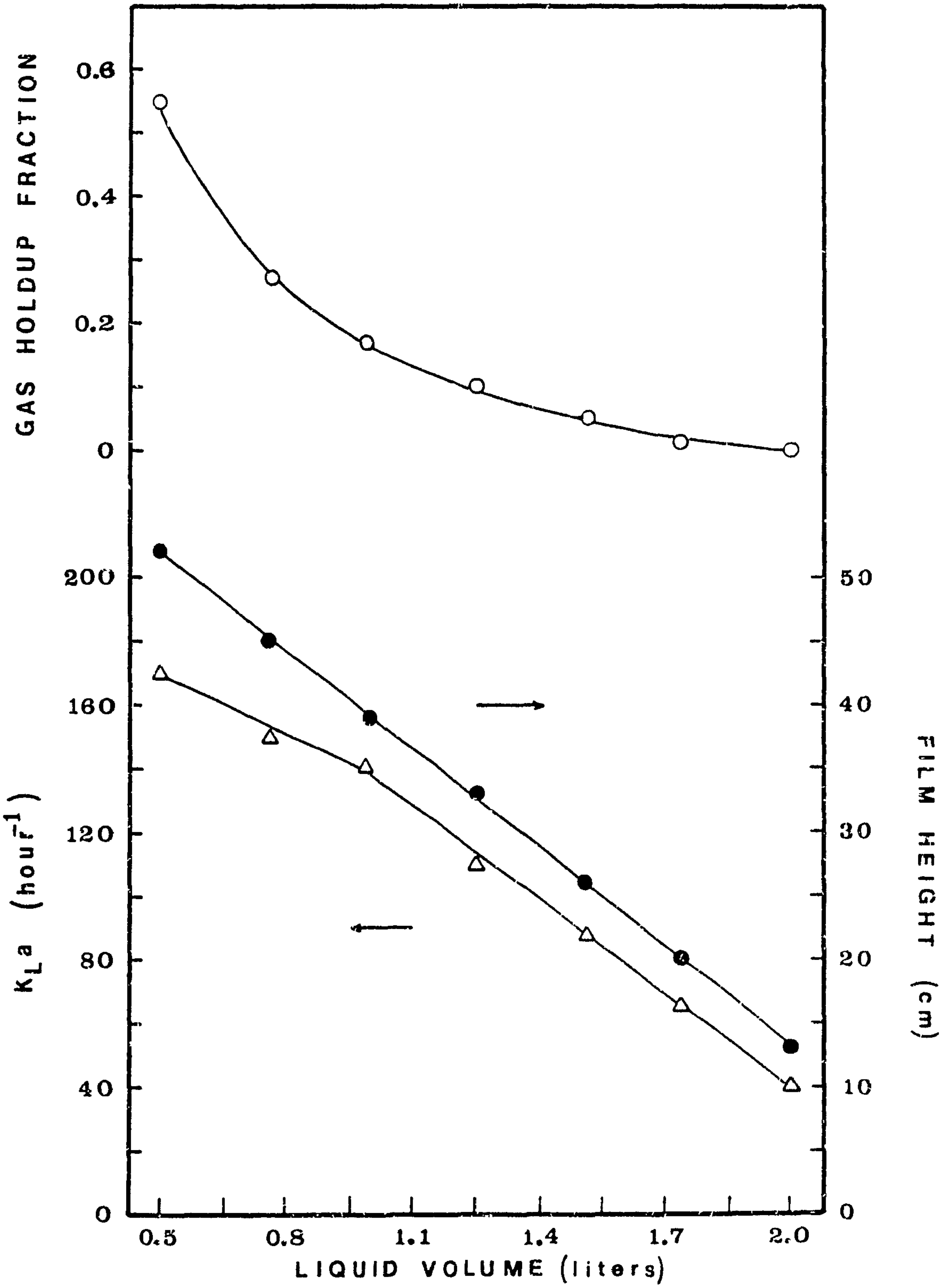
4.5.2 Results

Since oxygen transfer from the gas to the liquid phase is dependent on the driving force, film resistance and interfacial area, it seemed probably that the working liquid volume would have a significant effect on the rate of oxygen transfer by controlling interfacial area. As illustrated in Figure 44, in distilled water the volumetric oxygen transfer coefficient (K_La) is a strong function of liquid volume reaching a maximum of 170 per hour at a minimum volume of 500 mL. The K_La decreases almost linearly to a minimum of 40 per hour, corresponding to a 2 liter working volume, a minimum film height of 13 cm and no gas holdup. Both the film height and K_La increase by a factor of 4 as the volume is reduced by the same factor and the gas holdup fraction increases from zero to almost 0.56. The close correspondence between the film height and the K_La indicates that it is likely across the falling film where most of the oxygen transfer occurs. Each centimeter of film height provides an interfacial area of

Figure 44

The Effect of Working Liquid Volume on Oxygen Transfer into Distilled Water

Shown is the variation in gas holdup fraction (○), liquid film height (●) and volumetric oxygen transfer coefficient (△) as a function of the working volume in the cyclone column reactor.


GAS HOLDUP FRACTION
0.6
0.4
0.2
0
K_La (hour^{-1})
200
160
120
80
40
0
FILM HEIGHT (cm)
50
40
30
20
10
0
0.5
0.8
1.1
1.4
1.7
2.0
LIQUID VOLUME (liters)

about 24 cm^2 or a maximum of 1300 cm^2 with a 500 mL working volume. The interfacial area provided by the entrained bubbles is not known since this requires measurement of the bubble size distribution.

The effect of surfactants on the oxygen transfer is very pronounced, even when the macroscopic hydrodynamics seem to be changed very little. For example, Figure 45 illustrates the effects of dodecyltrimethylammonium bromide (DTAB), a cationic surfactant. At a concentration of 0.5 g/L, although there is no effect on film height or gas holdup, the K_La is significantly decreased, especially with low liquid volumes. Increasing the DTAB concentration to 1.0 g/L results in a further decrease in the surface tension (to 48 mN/m) and induces some foaming, thereby increasing the gas holdup while decreasing the film height. Oxygen transfer becomes almost independent of liquid volume, K_La remaining at a low of 20 per hour except at the highest volumes where there is a slight increase.

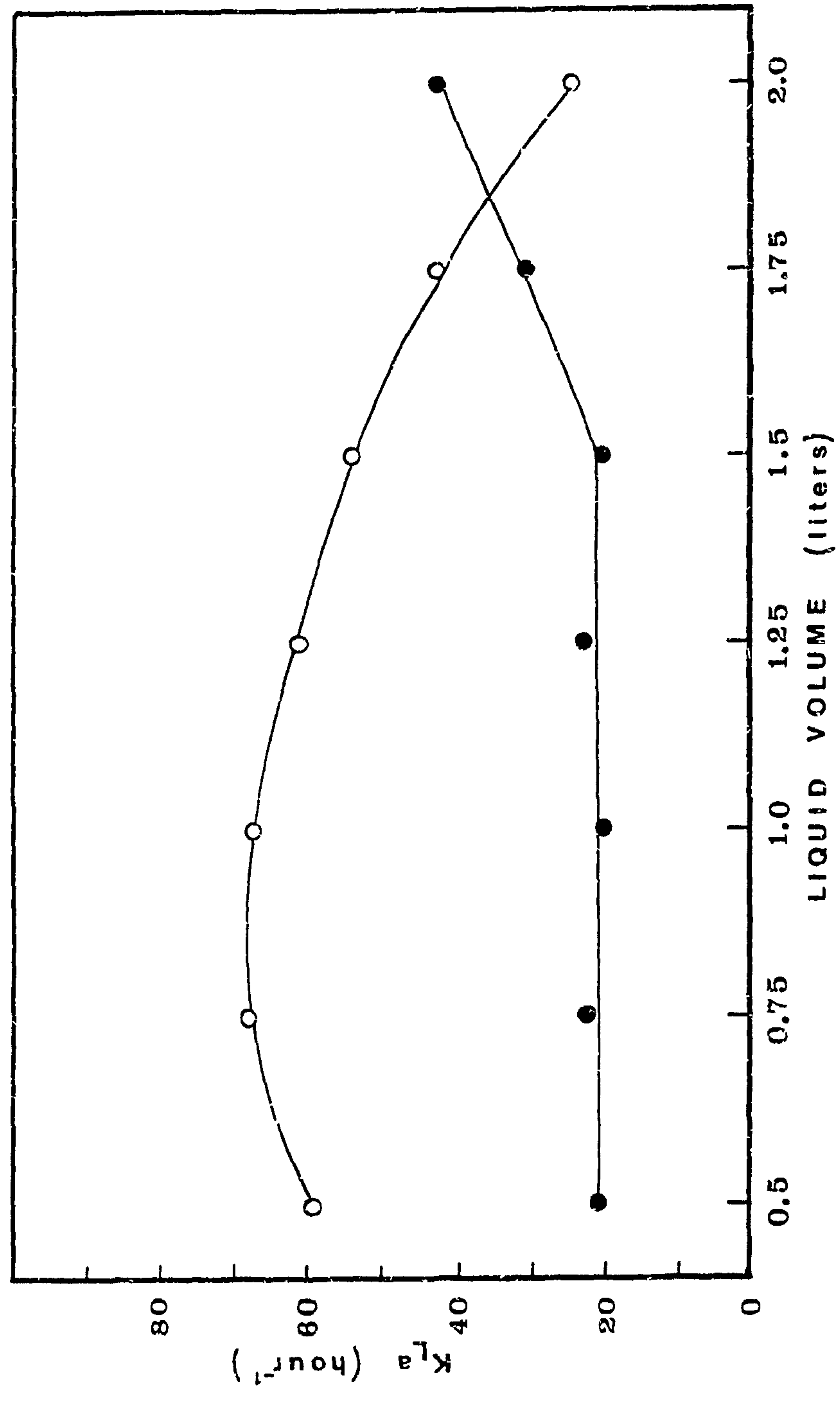
Similar trends are observed with sodium dodecylsulfate (SDS), an anionic surfactant (Figure 46), although foaming and, therefore, increased gas holdup occur even at a concentration of 0.05 g/L and a surface tension of 63 mN/m.

It was important to know if these effects were likely to be encountered during the production of a biosurfactant. A variety of oxygen transfer experiments were conducted on cell-free broth following a batch fermentation. The raw mineral

Figure 45

The Effect of DTAB on Oxygen Transfer

Shown is the variation in the volumetric oxygen transfer coefficient (K_La) as a function of liquid volume for two concentrations of dodecyltrimethylammonium bromide, 1.0 g/L (●) and 0.5 g/L (○).

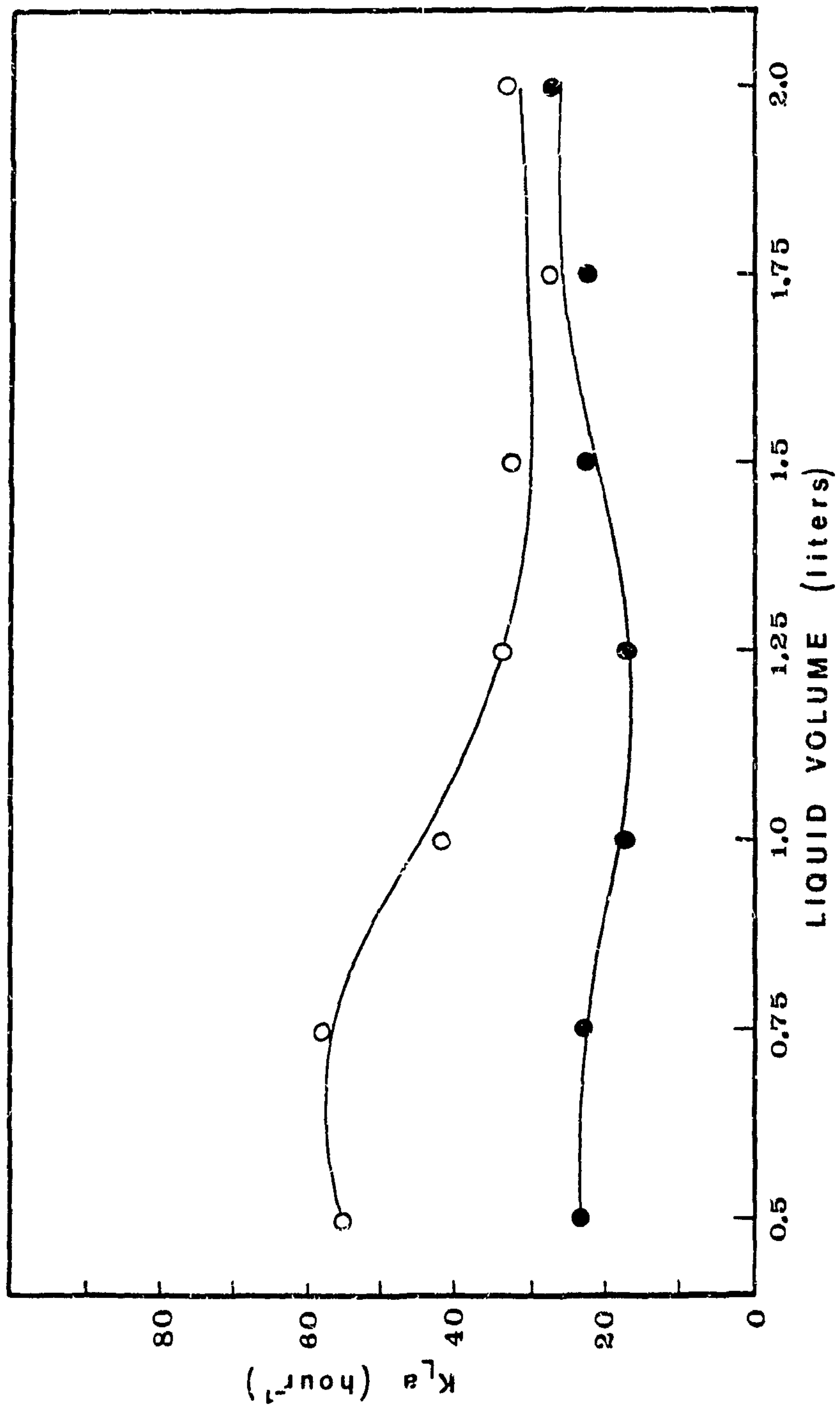


LIQUID VOLUME (liters)
K_La (hour^{-1})
0.5
0.75
1.0
1.25
1.5
1.75
2.0
0
20
40
60
80

Figure 46

The Effect of SDS on Oxygen Transfer

Shown is the variation in the volumetric oxygen transfer coefficient (K_La) as a function of liquid volume for two concentrations of sodium dodecylsulphate, 0.10 g/L (●) and 0.05 g/L (○).



K_La (hour^{-1})
0
20
40
60
80
0.5
0.75
1.0
1.25
1.5
1.75
2.0
LIQUID VOLUME (liters)

salts and glucose medium had no effect on oxygen transfer, giving results identical to those obtained on distilled water. However, the cell-free broth affected the K_La and hydrodynamics in the cyclone column quite dramatically as illustrated in Figures 47 and 48. With a one liter working volume a broth concentration as low as 1% (vol/vol) reduced the K_La to about 25 per hour even though the surface tension was over 50 mN/m. Further increases in proportion of broth to reach the critical micelle concentration (at a surface tension of about 30 mN/m) resulted in a minimum K_La of 10 per hour.

Previously it was shown by Cooper et al. (1981) that lowering the pH of the broth resulted in a loss of surfactant activity as monitored by surface tension measurements. Figure 49 illustrates that the same is true for the effects on oxygen transfer. There is a very sharp increase in the K_La as the pH is lowered from 6.2 to 5.7. However, the maximum effect on surface tension does not occur until between pH 5.2 to 4.1. In contrast to both the DTAB and the SDS, the gas holdup fraction is much less affected by the working volume of broth. This is due to the presence of a stable foam layer which is broken more easily by the increased shear at the low volumes. However, the very weak dependence of K_La on liquid volume again indicates the relative unimportance of gas holdup or film height in the presence of surfactants. It would, therefore, seem that the surfactants must affect the

Figure 47

The Effect of Cell-free Broth on Oxygen Transfer

Shown is the variation in the gas holdup fraction (○), the liquid film height (●) and the volumetric oxygen transfer coefficient (△) as function of the working volume of cell-free broth in the cyclone column reactor.

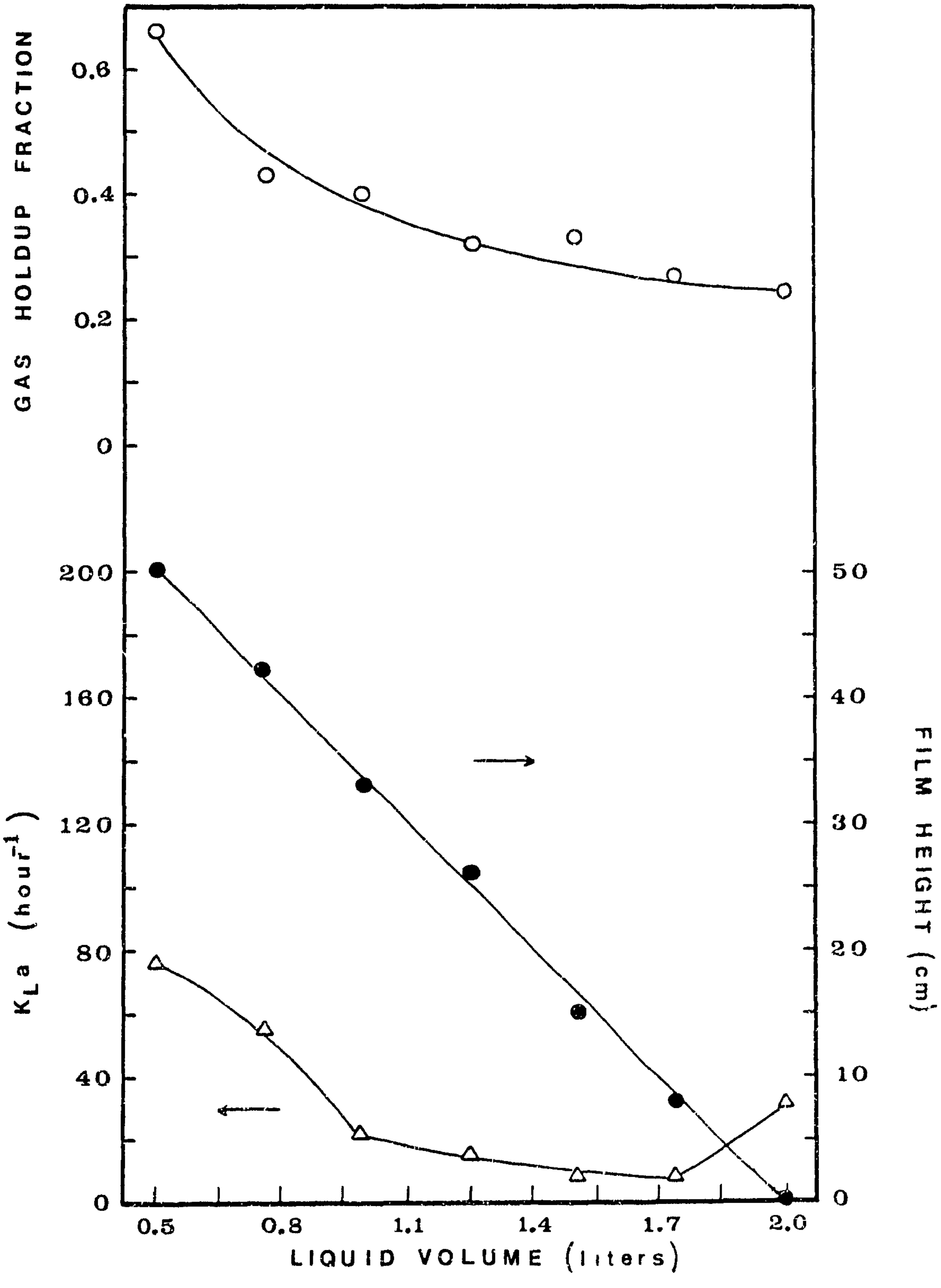

GAS HOLDUP FRACTION
0.6
0.4
0.2
0
K_La (hour^{-1})
200
160
120
80
40
0
FILM HEIGHT (cm)
50
40
30
20
10
0
0.5
0.8
1.1
1.4
1.7
2.0
LIQUID VOLUME (liters)

Figure 48

The Effect of Broth Concentration on Oxygen Transfer

Shown is the variation in surface tension (▲) and the volumetric oxygen transfer coefficient (△) as the cell-free broth was diluted with distilled water. Volume was constant at 1.0 liters.

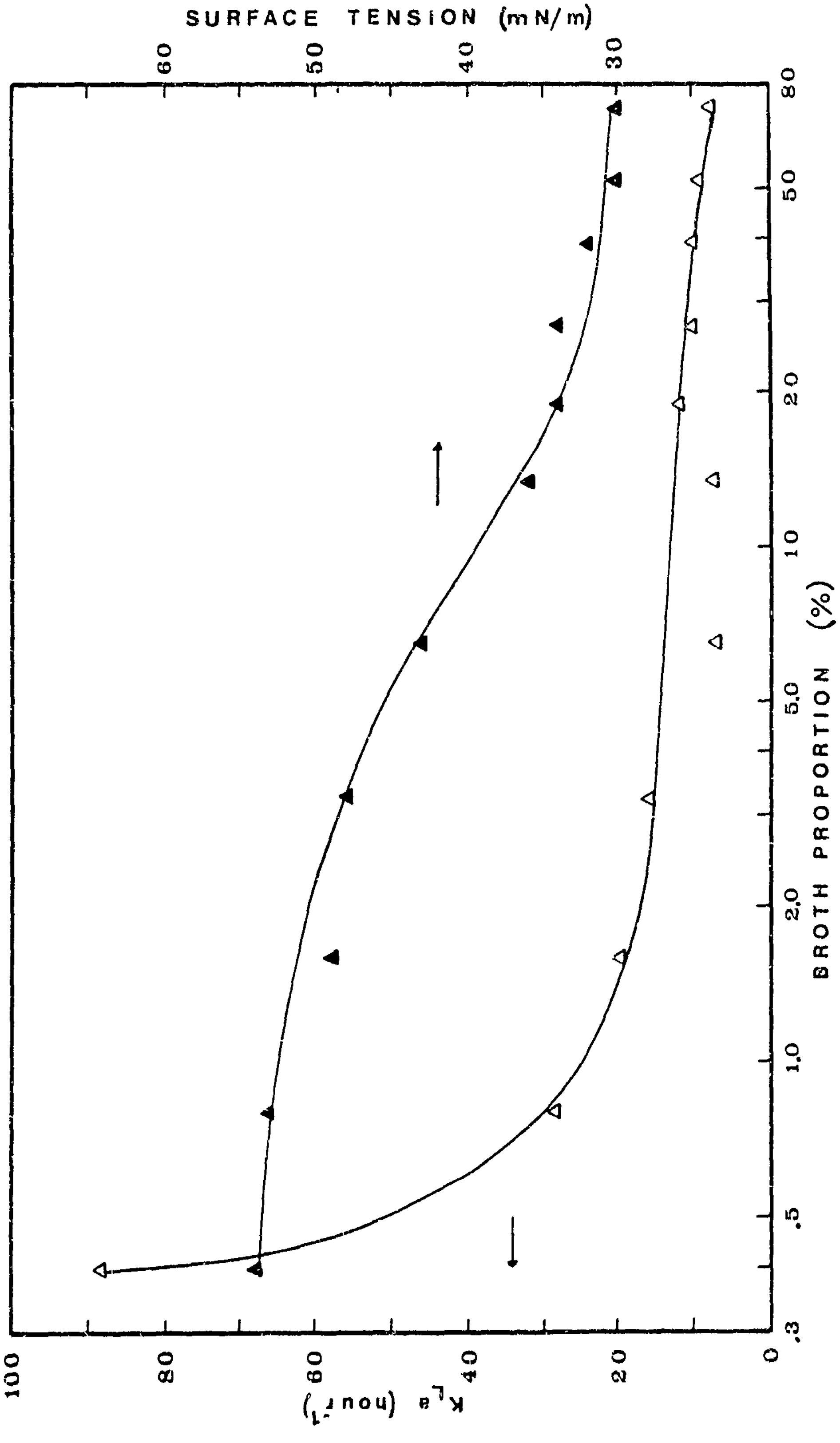
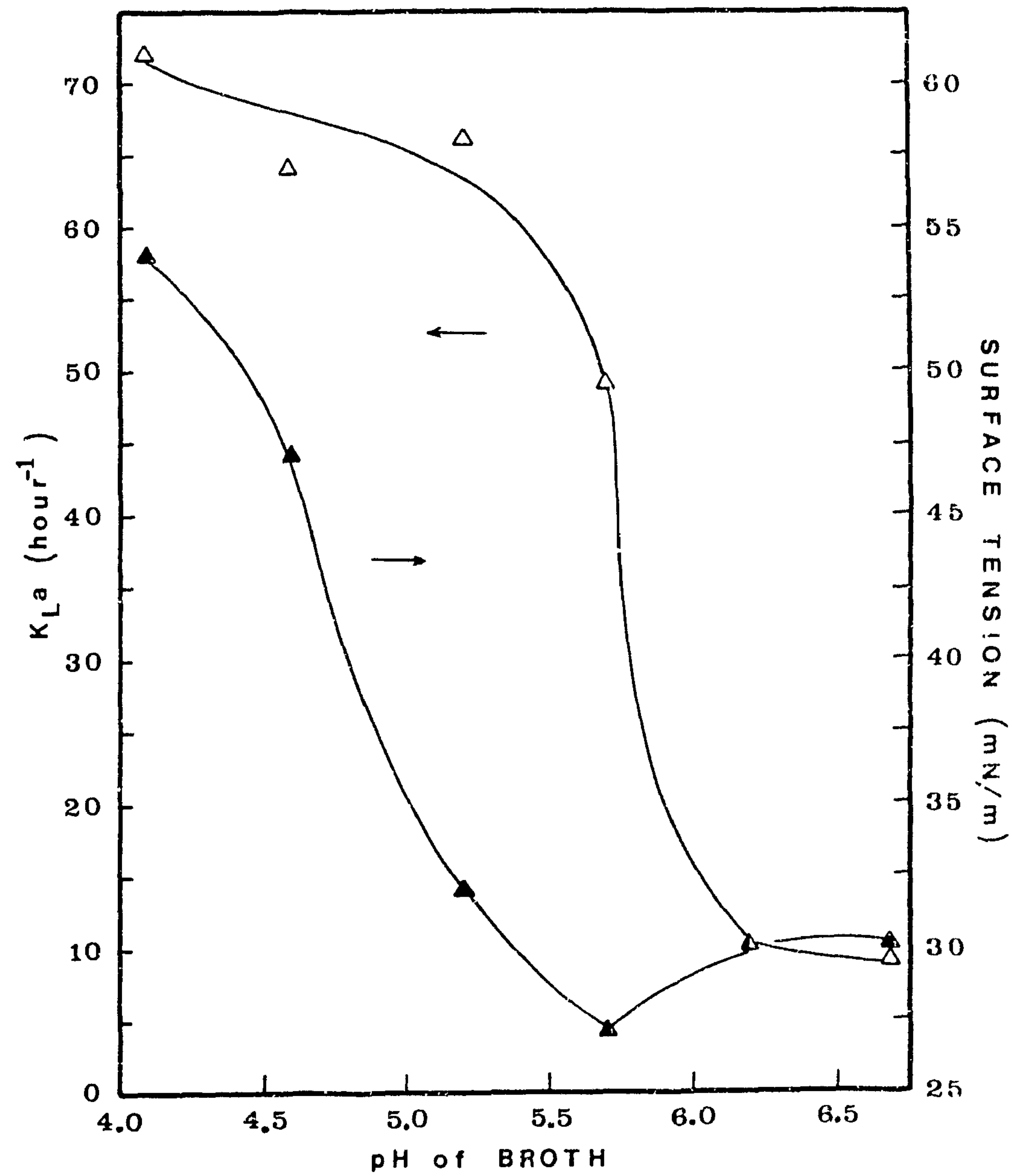

SURFACE TENSION (mN/m)
60
50
40
30
BROTH PROPORTION (%)
.3
.5
1.0
2.0
5.0
10
20
50
80
K_La (hour^{-1})
100
80
60
40
20
0

Figure 49

The Effect of Broth pH on Oxygen Transfer

Shown is the effect of changing the pH of cell free broth on the surface tension (▲) and volumetric oxygen transfer coefficient (△). Volume was constant at 1.0 liters.


70
60
50
40
30
20
10
0
K_La (hour^{-1})
60
55
50
45
40
35
30
25
SURFACE TENSION (mN/m)
4.0
4.5
5.0
5.5
6.0
6.5
pH of BROTH

interfacial film resistance and that this effect overwhelms any change in interfacial area as a result of increased bubble entrainment.

4.5.3 Discussion

The rate of oxygen transfer obtained in the cyclone column with distilled water compares favourably with conventional stirred tanks (Linela et al. 1987, Robinson et al. 1973), albeit at significantly higher power input. With a one liter working volume and 1 liter per minute aeration, the ration of $K_La/V_S^{0.4}$ (where V_S is the superficial gas velocity) is 0.37 $m^{-0.4}s^{0.6}$ which corresponds to about 1.5×10^3 watts per cubic meter of power input to a stirred tank (Linela et al. 1987). In the bench scale cyclone column a 0.018 horsepower centrifugal pump is used for mixing, resulting in a power consumption of about 1.4×10^4 watts per cubic meter, or nearly ten times higher than in a stirred tank. Increasing the reactor volume to two liters but maintaining the same pumping rate results in an almost 4 fold decrease in K_La due to the decreased film height. However, increased power economy could be achieved by increasing column height as the volume is increased, thereby maintaining adequate film height. Depending on the pump, this would result in only a small increase in power consumption due to the increased pressure drop in the longer recirculation loop. The higher pump discharge pressure would be compensated by the increased head on the suction side of the pump. The effect of recirculation rate on oxygen transfer was not studied, however, this would likely have a significant influence on the hydrodynamics, especially the film thickness on the inside of the column and the amount of entrained bubbles due to vortexing. These parameters should be investigated prior to scale-up where power becomes a more important concern than with the bench scale units.

The effects of the various surfactant solutions tested in the cyclone column can be seen on both the large scale hydrodynamics (gas holdup) and on the film resistance. Except in the case of 0.5 grams per liter of DTAB there is an increase in gas holdup when compared to distilled water, yet this is accompanied by very significant decreases in the K_La. This indicates a much larger effect on K_L that overcompensates for any increase in interfacial area. The effect on K_L is so large as to make the rate of oxygen transfer essentially independent of the working liquid volume. From Figure 18 it can be seen that even though the K_La can be reduced to as low as 10 per hour, there is sufficient capability to grow B. subtilis at about 0.6 grams per liter. At low surface tensions (less than 30 mN/m) at least 75% oxygen saturation is maintained in the broth. This would indicate that oxygen limitation may begin if the biomass concentration exceeded about 2.5 grams per liter, corresponding to a limiting concentration of $(NH_4)_2HPO_4$ of about 0.016 molar. Since the effects of the surfactants are primarily on interfacial film resistance, then it is likely that equivalent performance would be achieved in a conventional stirred tank.

4.6 Surfactin Production

4.6.1 Procedure

The metabolism of the cells, as reflected by growth and surfactant production, is a function of both the nutrient environment and the temporal nature of this environment. As previously discussed, the use of continuous phasing reveals the cellular metabolism in terms of the cell division cycle as a result of the temporal correspondence between cell division and the nutrient environment. Feedback phasing allows an accurate and consistent correspondence between the time of exhaustion of the limiting nutrient and introduction of a fresh nutrient supply, thereby eliminating variations in the period of cell starvation prior to the subsequent cell cycle. This is particularly useful when investigating the effects of changes in the composition of the nutrient medium because of the automatic adjustment of the period of nutrient addition to changes in the specific growth rate.

Previous work by Cooper et al. (1981) had strongly implicated the importance of manganese and iron for both the growth and production of surfactant by B. subtilis. However, conventional batch culture techniques were used and the results were mostly qualitative. It remained unclear whether surfactant production was affected by these ions or whether the effects were only on growth rate. To clarify these effects feedback phasing was implemented with media varying in both iron and manganese concentrations. The growth rate was automatically obtained from the time between nutrient cycles and the quantity of surfactant production was measured by the technique of serial dilutions of the broth, as described in Appendix A. Both the nitrogen and carbon concentrations in the media were increased in comparison to the nitrogen limited medium as described in Table 1. The $(NH_4)_2HPO_4$ concentration was increased to 0.006 molar and the glucose concentration to 15 grams per liter. The increased surfactant concentrations resulted in greater foam production. This was somewhat alleviated by the use of an additional recirculation pump and a separate foam collection vessel.

4.6.2 Results

The results from the tests of six different media are presented in Table 12. In each case the experiments were started from a batch culture and operated for a sufficient number of generations of cells to ensure representative data (on average about 30). Separate analyses of the samples obtained at the end of the batch cultures indicated that the

Table 12

The Effects of Manganese and Iron on Surfactin Production

#	Nutrient concentrations (molar) Mn	Fe	Average T_d (min)	CMC^{-1}	Production* (mg/L-hour)
1)	3×10^{-4}	2.0×10^{-4}	119	9	23
2)	3 x 10-4	1.0×10^{-4}**	177	8	14
3)	8 x 10-5	1.25×10^{-4}	75	6	24
4)	3×10^{-5}	1.0×10^{-4}**	90	7	23
5)	1.3×10^{-5}	1.0×10^{-4}**	94	4.5	14
6)	0	2.0×10^{-4}	215	-	-

* based on a critical micelle concentration of 10 mg/L

** iron limited medium.

surfactant concentration was approximately one half of that obtained while continuously phasing the cells. For example, with medium 4 the reciprocal of the critical micelle concentration (CMC^{-1} as described in Appendix A) was 3.6 following the batch culture, then increasing to about 7 during phasing.

The manganese and iron concentrations appear to significantly affect specific growth rate but have only a slight influence on the concentration of surfactant. This was true even with media 2, 3 and 4, which were actually iron limited as a result of the increased level of nitrogen. Although increasing either manganese or iron generally resulted in faster growth, this was not the case when iron was limiting. As the manganese was reduced from 3×10^{-4} molar to 3×10^{-5} molar with 1.0×10^{-4} molar iron, the doubling time decreased from 177 to 90 minutes. This may explain why the shortest doubling time, 75 minutes, was obtained at relatively low concentrations of both iron and manganese. With only 1.25×10^{-4} molar iron, the medium was just barely nitrogen limited due to a small excess of iron. Since manganese appears to affect growth differently depending on the relative amounts of iron and nitrogen, operating at the point where both iron and nitrogen are close to limitation may eliminate the effects of manganese. This may also explain the total lack of surfactant production with medium 6. In contrast, the medium of Cooper et al. (1981) results in levels of surfactant comparable to most of the media tested, even though, like medium 6 it has no manganese. However, the iron is considerably lower, at 4.0 x 10^{-6} molar, and the nitrogen concentration is higher. Therefore, it is likely to be iron limited, which unlike nitrogen limitation, requires low concentrations of manganese for good growth.

4.6.3 Discussion

The production rate of surfactin can be increased by increasing either the concentration of product or the rate of throughput per unit volume. Since all the evidence indicates that surfactin is a growth associated product, it is likely that the maximum rate of production will occur if the cells are growing at a maximum rate and if the concentration in the broth is the maximum that can be supported. This assumes that the medium is appropriate for inducing surfactin production. It has been shown that some nutrient combinations do not seem to be suitable.

An important factor in the production of surfactin, observed throughout this study, is the effects of a nutrient limitation. Surfactin appears to be produced in response to an environmental stress that occurs as the limiting nutrient is exhausted in the medium. This explains why production occurs during the last generations of the logarithmic growth phase in batch culture. Continuous phasing, therefore, seems to be ideally suited to a product of this type since a nutrient limitation occurs during every generation of cells. This results in approximately a 100% increase in the concentration of surfactin in the broth when compared to a batch culture with the same medium. However, nutrient limitation can also inhibit or repress surfactin production if the period of nutrient starvation is excessive. Minimizing this period with feedback control maximizes the number of successive generations that will continue to produce high levels of surfactin.

The cell culture technique affects the dynamics of the nutrient environment, which has subsequent effects on the cellular metabolism. It is well known that a lag period occurs prior to the start of cell division in a batch culture. This is also generally true in continuous phasing. The dramatic discontinuity that occurs in the environment at the time of nutrient dosing results in a lag in growth at the beginning of each cell cycle. Therefore, the maximum specific growth rate that occurs during the logarithmic growth phase of a batch culture cannot be obtained during continuous phasing. Again this problem is greatly alleviated by the use of feedback control to minimize the period of nutrient exhaustion at the end of the cell cycle. This minimizes the lag at the beginning of the subsequent cycle such that the specific growth rate becomes essentially identical to that which can be achieved in batch culture. For example, the minimum biomass doubling time in a medium limited by 2 x 10^{-3} molar $(NH_4)_2HPO_4$ was 145 minutes in batch culture and about 150 minutes in continuous phasing.

The use of the feedback control system for timing the nutrient dosing maximizes both the specific growth rate and the number of successive generations producing surfactin. The advantage in productivity in comparison to batch can be calculated as follows:

$$R_p = ((T_B/T_d) \times (C/C_B))/2 \qquad (10)$$

where R_p is the rate of production; T_B is the total time for a batch culture; T_d is the doubling time of the biomass during continuous phasing; C is the concentration of surfactin obtained while phasing and C_B is the concentration of surfactin obtained at the end of the batch. The factor '2' is required to account for the fact that only one half of the broth is harvested each cycle while phasing. Data obtained from growth on medium 4 results in the following value for R_p:

$$R_p = ((16/1.5) \times 7/3.6))/2$$
$$= \underline{10.4}$$

This means that the use of continuous phasing increases the production rate of surfactin by more than a factor of ten in comparison to batch culture. In practice, batch culture has additional disadvantages, such as the time required for cleanup between batches and preparation of the inoculum which will further improve the relative advantage of continuous phasing.

No experiments were conducted using the continuous chemostat technique. However, it is unlikely that productivities equivalent to those obtained with continuous phasing could have been achieved. Operations of a chemostat at a dilution rate approaching the maximum specific growth rate is a very unstable situation that can easily lead to cell washout. Also, the concentration of surfactin that could be obtained is unknown, but, since the basis of a chemostat is that no absolute nutrient exhaustion occurs, production may be inferior to that occurring in continuous phasing where the environmental stress is more severe.

The maximum concentration of surfactin that was obtained in this study was about 90 mg/L, corresponding to a CMC^{-1} of 9. This was produced with a biomass concentration of about 800 mg/L. This relatively low concentration of bacteria could be increased by simply increasing the concentrations of the nutrients in the medium. In this way the overall production rate of surfactin could also be increased. However, other process considerations then become important. High concentrations of surfactin tend to be associated with poor oxygen transfer, high levels of foam and excessive power requirements for recirculation of the broth. Overcoming these problems may require extensive equipment modifications or completely new designs. These considerations are beyond the scope of this study and, therefore, it is not possible to predict the maximum rate of surfactin production or associated costs. However, it can be stated with confidence that continuous phasing with feedback control will be significantly less expensive than any batch process.

CHAPTER 5

SYNTHESIS AND CONCLUSIONS

The history of the cultivation of microorganisms describes a gradual increase in the understanding and appreciation for the metabolic phenomena that underlie the growth of a large population of cells. The progress in understanding was accelerated with the development of the chemostat technique for continuous cultivation by Monod (1950). For the first time the experimenter was able to define growth in terms of an environmental parameter, the concentration of a rate-limiting nutrient. In doing so, some predictability and control over cellular metabolism became possible. Although a great advancement over batch culture, the chemostat technique could only reveal responses of the population averaged over the entire cell cycle. It was the perception and appreciation of cellular metabolism as dynamic, cyclical phenomena by Goodwin (1963, 1966) and others, that led to the development of continuous phasing by Dawson (1965, 1969). The periodic addition of a growth-limiting nutrient was able to entrain the cellular metabolism, thus revealing cell cycle events. It became possible to relate environmental factors directly to temporal events in the cell cycle. However, interpretation of the metabolic responses was not easy because of the interaction between the imposed period of the nutrient cycle and the resulting cell cycle. Above a certain minimum, selection of a time period for the nutrient cycle was largely arbitrary. Other difficulties, encountered particularly in this study with Bacillus subtilis, included the de-stabilizing effects of the period of nutrient starvation and changing cell performance over successive generations.

This has led, in this study, to the development of a feedback system to control the period of nutrient cycling based on the metabolic requirements of each generation of cells. The feedback is based on the continuous measurement of the concentration of dissolved oxygen in the broth which is proportional to the respiration rate of the cells. At the time of nutrient exhaustion in the broth, there is a rapid increase in the concentration of dissolved oxygen. This information is used as the key parameter for determining the appropriate time for the addition of fresh nutrients. The hardware required to implement the feedback control includes a dissolved oxygen probe, a multi-meter, a data acquisition and control interface (A/D and D/A conversion), a computer programmed in HP Advanced Basic and the standard solenoid valves. A total of 12 computer programmes were written to explore various experimental possibilities that emerged as a result of this new development in cell culturing.

Previous work in batch culture and using the conventional technique for continuous phasing, had implicated surfactin production with the occurrence of a nutrient limitation.

Since the time of exhaustion of the growth-limiting nutrient could now be automatically determined for each successive generation of cells, the importance of this occurrence was investigated further. Continuous operation with feedback control and a minimal period of nutrient starvation in each cycle, resulted in the maximum productivity of surfactin. The apparently inevitable inhibition or suppression of surfactin production was delayed for about 80 generations of cells, greatly exceeding the results obtained when a specific period of nutrient dosing had been imposed on the population. Subsequent experiments confirmed that the addition of a period of starvation at the end of the cell cycle resulted in a rapid decrease in surfactin production. Thus, it appeared that surfactin could be produced as a result of a general response to stress imposed by the limitation of an essential nutrient. This response eventually disappeared during the continued application of this stress over successive generations.

Throughout the course of this study it became clear that any interpretation of cell-environment interactions should account for both the metabolic responses of individual generations and the metabolic history of previous generations. A single generation of cells, if studied outside of its historical context, will only provide a limited view of a wide range of possible responses. This observation is very important when trying to interpret data from batch cultures, or in any situation where responses of successive generations of cells are being studied. Because of the tendency for living systems to continually adapt to the external environment, it is unlikely that successive generations of cells ever respond exactly the same as preceding generations. Constant metabolic performance can apparently be approached for finite periods of time when the environment is maintained constant, as occurs in a chemostat. However, it seems that more insight into cellular metabolism can be obtained by subjecting the cells to a dynamic environment. The use of feedback control and continuous phasing allowed the dynamic response of B. subtilis to be studied when subjected to step changes in the concentration of manganese, without the danger of losing process stability. Based on these experiments, growth of B. subtilis was revealed as a stable limit cycle, with the effects of manganese on the specific growth rate modulated over several generations of cells. Further development of this technique could provide a rapid method for optimization of the growth medium.

From the engineering perspective, continuous phasing with feedback control offers many potential cost advantages in comparison to conventional batch culturing. In addition to an overall increase in rate of production by at least a factor of 10, the process will maintain stability even during changes in the quality of the medium and after brief upsets, for example, during power failures. Unlike a batch culture with its inherent sensitivity to the metabolic condition of the inoculum, production rates in a continuous process can be predicted with confidence. As a result, cost estimates and production planning can be more realistic.

The following are the conclusions of this study:

i) Batch culturing of B. subtilis ATCC 21332 resulted in reproducible specific growth rates during the logarithmic phase, however, the duration of the lag period was variable. Generally, surfactin production became significant during the growth of the final generations of cells.

ii) An improvement over the convential method of batch culturing can be made if several batches are operated consecutively. This provides an inoculum that is more uniform for each batch and allows the results to be interpreted over more generations of cells that have been subjected to a similar environment.

iii) The use of continuous phasing with imposed periods of nutrient cycling, revealed two significant aspects of surfactin production: the importance of nutrient limitation and the lack of consistent metabolic performance over successive generations of cells. Interpretation of the results was difficult due to the interaction between the nutrient cycles and the cell cycles. The technique lacked the level of control necessary to further investigate these phenomena.

iv) The addition of feedback control to the standard technique for continuous phasing greatly improved the utility of the method. Since a period of nutrient cycling is no longer imposed on the cells, the measured responses are a direct reflection of the interaction between the cells and the composition of the medium.

v) By minimizing the period of nutrient starvation with the feedback control system, much greater consistency in metabolic performance is achieved and surfactin production can be maintained for at least 80 consecutive generations of cells.

vi) Extending the period of nutrient starvation results in a more rapid decline in surfactin production. It is possible that surfactin is produced in response to environmental stress and this response is eventually suppressed by repeated starvation during successive generations of cells.

vii) Surfactin production can be restored by growing the cells on nutrient agar prior to inoculation in batch culture. This indicates that continuous phasing does not result in irreversible genetic changes in the population.

viii) The dynamic response of B. subtilis ATCC 21332 to step changes in the residual manganese concentration, indicates that cell growth can be modelled as a stable limit cycle.

ix) Continuous phasing with feedback control will increase the production rate of surfactin by at least a factor of 10 in comparison to batch culturing.

x) Surfactants significantly decrease the rate of oxygen transfer in the cyclone column reactor, likely by increasing the diffusional resistance across the gas-liquid film. Process design for biosurfactant production must consider this factor to ensure adequate availability of oxygen for the growth of the cells.

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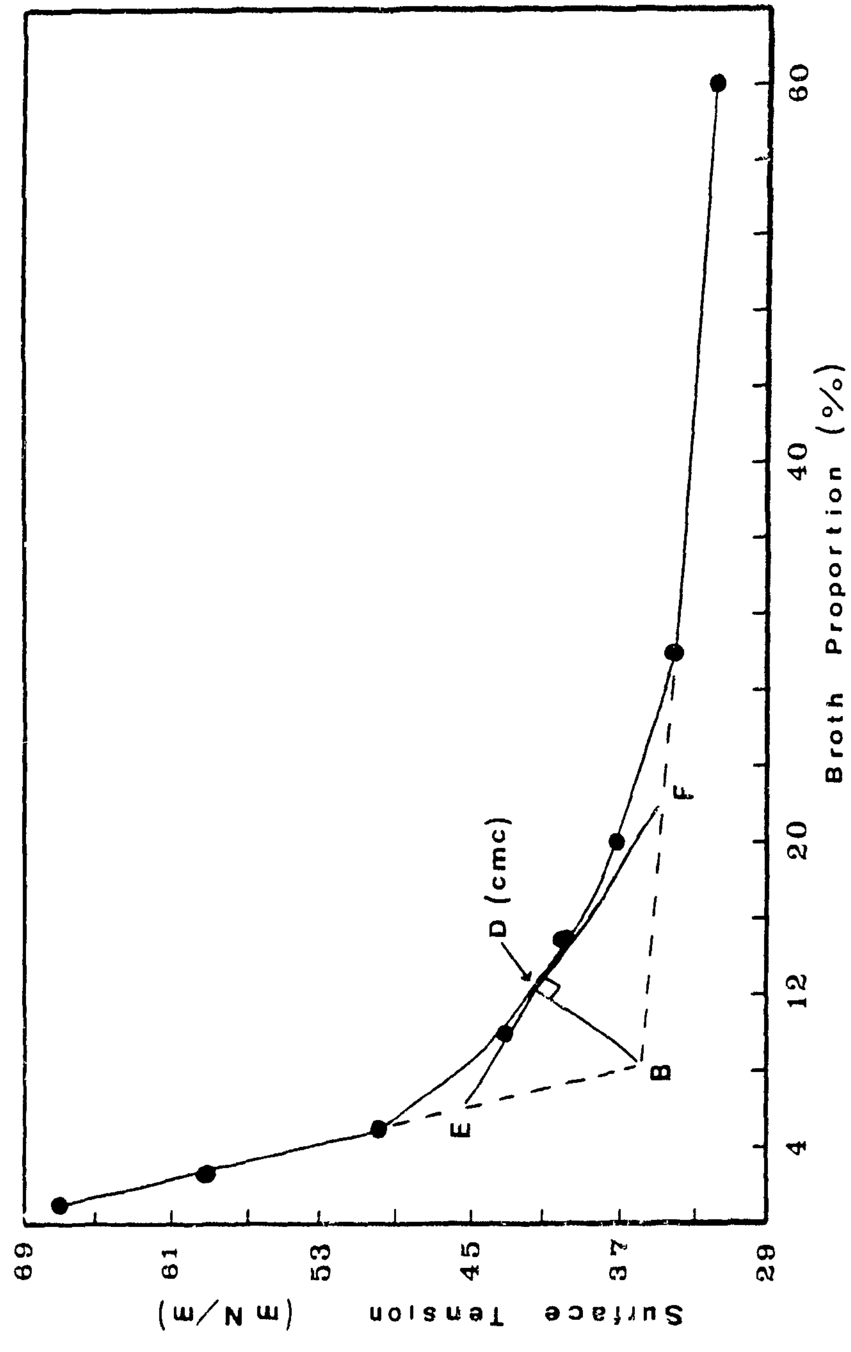
APPENDIX A

METHOD FOR QUANTIFYING SURFACTANT CONCENTRATION FROM MEASUREMENTS OF SURFACE TENSION

Theoretically, the critical micelle concentration (CMC) can be determined by plotting surface tension as a function of surfactant concentration (from dilutions of the broth), since the slope of the curve changes abruptly at the CMC. However, the rate of change in the slope of the curve is a function of both the particular surfactant and the type and quantity of impurities in the system. Usually there is a range of concentrations over which the slope of the curve changes from a steep non-micellar form to a relatively flat line where micelles predominate. The transition point on the curve is defined as the critical micelle concentration (CMC) and is determined graphically as depicted in Figure 50. The surface tension of cell-free broth was measured after performing a series of dilutions with distilled water. After plotting the data, tangents are drawn along the two straight portions to interact at point B. Then, a line is drawn from B to intersect the tangent E-F at 90 degrees. The intersection point, D, is defined as the CMC. The dilution factor required to reach the CMC is a measurement of the quantity of surfactant present in the original broth and is represented by CMC^{-1}.

Figure 50

A Method for Determining the Critical Micelle Concentration


Surface Tension (m N/m)
69
61
53
45
37
29
Broth Proportion (%)
4
12
20
40
60
D (cmc)
E
B
F

APPENDIX B

DETERMINATION OF K_La BY THE SULPHITE OXIDATION METHOD

This method has been widely accepted as a standard technique for characterizing gas-liquid contactors (Yoshida et al. 1960, Hyman and Van Den Bogaerde 1960) and has been used for verifying the accuracy of dynamic methods using oxygen probes (Linek et al. 1987). Several oxidations were performed in the cyclone column with a one liter working volume of 0.5 molar sodium sulphite catalyzed with 15 ml of 0.2 M $CuSO_4$. The oxidations were allowed to continue for 2 hours with aeration at either 0.17 or 1.0 liters per minute and samples obtained every 15 minutes. The rate of formation of sulphate ions was monitored by adding a measured sample volume to a standardized solution of iodine. The sulphite ions remaining in the sample were converted to sulphate by the iodine according to the following equation:

$$Na_2SO_3 + I_2 + H_2O \rightarrow Na_2SO_4 + 2\ HI \tag{11}$$

The amount of iodine consumed by the reaction with the sulphite ions was determined by a back titration with sodium thiosulphate which converted the remaining iodine by the following equation:

$$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaI \quad (12)$$

Since 126.06 g of Na_2SO_3 are required to reduce 253.8 g of I_2

$$\Delta I_2/\Delta t = 2.014\ \Delta Na_2SO_3/\Delta t \quad (13)$$

and the moles of Na_2SO_3 oxidized per unit time:

$$QSO_3 = -((\Delta I_2/\Delta t)/(2.014 \times 126.06)) \times (1000/2) \quad (14)$$

where 1000/2 is the ratio of the total volume in the reactor to the sample volume. Therefore, for a 2 ml sample and a 1 liter working volume:

$$QSO_3 = -1.971 \times (\Delta I_2/\Delta t) \quad (15)$$

and

$$QO_2 = -0.986 \times (\Delta I_2/\Delta t) \quad (16)$$

Since $QO_2 = K_l a\ (C^* - C_L)$ and C_L is zero;

$$K_l a = (-0.986 \times (\Delta I_2/\Delta t))/C^* \quad (17)$$

where C* is the concentration of dissolved oxygen at the interface. At 30°C, C* will equal 0.00023 moles per liter.

APPENDIX C

COMPUTER PROGRAMME LISTINGS IN HP BASIC

```
[illegible] *** [illegible] *** [illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible] HOURS, MINUTES, SECONDS SINCE MIDNIGHT
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]                                          [illegible]
[illegible]
[illegible]
[illegible] : "[illegible] AT",HR
[illegible]
[illegible]
[illegible] OUTPUT [illegible]                        MEASURE INITIAL WEIGHT
110 ENTER [illegible]
[illegible]                                           CONVERT [illegible]GE
[illegible]                                           CALCULATE DESIRED WEIGHT
1[illegible] OUTPUT 70[illegible] ;"[illegible]"      OPEN MEDIUM VALVE
1[illegible] OUTPUT [illegible] ;"[illegible]"        MEASURE NEW WEIGHT
1[illegible] ENTER [illegible] ; WT[illegible]        ASSIGN TO WT[illegible]
[illegible] IF WT[illegible] THEN GOTO [illegible]    COMPARE WEIGHTS
[illegible] GOTO 1[illegible]                         RETURN TO START OF LOOP
2[illegible] OUTPUT 70[illegible] ,"[illegible]"      CLOSE MEDIUM VALVE
[illegible] GOTO [illegible]                          PROGRAM[illegible] [illegible]ES
[illegible] TIMER# 1
[illegible] ON TIMER# 1,CYCLE GOTO [illegible]        RESET TIMER FOR NEXT CYCLE
[illegible] OUTPUT 70[illegible] ;"[illegible]"       MEASURE WEIGHT
[illegible] ENTER 70[illegible] WT[illegible]         ASSIGN TO WT[illegible]
[illegible] WT[illegible]                             CALCULATE DESIRED WEIGHT
[illegible] OUTPUT 709 ;"[illegible]"                 OPEN HARVESTING VALVE
[illegible] OUTPUT [illegible] ,"[illegible]"         MEASURE NEW WEIGHT
[illegible] ENTER [illegible] WT[illegible]
[illegible] IF WT[illegible] THEN GOTO [illegible]    COMPARE WEIGHTS
[illegible] GOTO [illegible]                          RESTART HARVESTING LOOP
[illegible] OUTPUT [illegible] , "[illegible]"        CLOSE HARVESTING VALVE
[illegible]40 OUTPUT [illegible] ;"[illegible]"       OPEN DOSING VALVE
[illegible] WAIT [illegible]                          WAIT ONE MINUTE
[illegible] OUTPUT [illegible] ,"[illegible]"         CLOSE DOSING VALVE
[illegible]                                           INCREMENT SAMPLE VALVE #
[illegible] IF [illegible] THEN GOTO [illegible]
[illegible]
[illegible] IF [illegible] 1 THEN GOTO [illegible]
[illegible] OUTPUT [illegible] , "[illegible]1"       OPEN SAMPLE VALVE #1
[illegible] WAIT [illegible]                          WAIT ONE MINUTE
[illegible] OUTPUT [illegible] , "[illegible]1"       CLOSE SAMPLE VALVE #1
[illegible] GOTO [illegible]
[illegible] IF [illegible] THEN GOTO [illegible]
[illegible] OUTPUT [illegible] ; "[illegible]"        OPEN SAMPLE VALVE #2
[illegible] WAIT [illegible]                          WAIT ONE MINUTE
[illegible] OUTPUT [illegible] , "[illegible]"        CLOSE SAMPLE VALVE #2
[illegible] GOTO [illegible]
[illegible] OUTPUT [illegible] ; "[illegible]1"       OPEN SAMPLE VALVE #
[illegible] WAIT [illegible]                          WAIT ONE MINUTE
```

```
[illegible]
[illegible] ICOUNT=ICOUNT [illegible]
[illegible] DISP USING [illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible] END
```

```
[illegible]

                                        PROGRAMME IDLES

                                        RESET TIMER FOR NEXT CYCLE
                                        MEASURE WEIGHT
                                        ASSIGN TO DATA
                                      CALCULATE DESIRED WEIGHT
                                        OPEN HARVESTING VALVE
                                        MEASURE NEW WEIGHT

                                        COMPARE WEIGHTS
                                        RESTART HARVESTING [illegible]
                                        CLOSE HARVESTING VALVE

                                        OPEN DOSING VALVE
                                        WAIT ONE MINUTE
                                        CLOSE DOSING VALVE
                                        INCREMENT SAMPLE [illegible]

                                        OPEN SAMPLE VALVE #1
                                        WAIT ONE MIN [illegible] SEC
```

```
430 OUTPUT 707 ;"OF[illegible]"            [illegible]
440 GOTO 5[illegible]0
450 IF S[illegible] E [illegible] THEN GOTO 500
460 OUTPUT 707 ;"[illegible]"              [illegible]
470 WAIT 2000                              [illegible]
480 OUTPUT 709 ;"OF[illegible]"            [illegible]
490 GOTO [illegible]
500 OUTPUT 7[illegible] ;"CL[illegible]1"  [illegible]
510 WAIT 2[illegible]                      [illegible]
520 OUTPUT 707 ;"[illegible]1"             [illegible]
530 CCOUNT=CCOUNT+1                        [illegible]
535 DISP USING 540 ;"[illegible]"          [illegible]
540 IMAGE 17A,DD,[illegible]
545 !
550 GOTO 1[illegible]                      [illegible]
560 END
```

```
[illegible]

                                   MEASURE INITIAL OXYGEN

                                   WAIT   MINUTES
                                   MEASURE NEW OXYGEN

                                   CALCULATE OXYGEN DIFFERENCE

                                   MEASURE WEIGHT
                                   ASSIGN TO WTIA
                                   CALCULATE DESIRED WEIGHT
                                   OPEN HARVESTING VALVE
                                   MEASURE NEW WEIGHT

                                   COMPARE WEIGHTS
                                   RESTART HARVESTING LOOP
                                   CLOSE HARVESTING VALVE


                                   OPEN DOSING VALVE
```

```
[illegible] WAIT [illegible]                                  [illegible]
[illegible] OUTPUT [illegible]                                [illegible]
[illegible]                                                   [illegible]
[illegible] IF [illegible]
[illegible]
[illegible] IF [illegible]
[illegible]                                                   [illegible]
[illegible] WAIT [illegible]                                  [illegible]
[illegible] OUTPUT [illegible]                                [illegible]
140 GOTO [illegible]
[illegible] IF [illegible]
460 OUTPUT [illegible]                                        [illegible]
[illegible] WAIT [illegible]                                  [illegible]
180 OUTPUT [illegible]                                        [illegible]
[illegible] GOTO [illegible]
[illegible] OUTPUT [illegible]                                [illegible]
510 WAIT [illegible]                                          [illegible]
[illegible] OUTPUT [illegible]                                [illegible]
[illegible] OUTPUT [illegible]                                [illegible]
[illegible]
[illegible]
[illegible]
[illegible]
545 [illegible]                                               [illegible]
550 [illegible]
[illegible] END
```

```
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]                                                  [illegible] VOLUME TO VOLTAGE
[illegible]
[illegible]
[illegible]
[illegible] HOURS, MINUTES, SECONDS SINCE MIDNIGHT
[illegible]
[illegible]
[illegible]
[illegible]
[illegible]
[illegible] ,D. [illegible]
[illegible]
[illegible]
[illegible]                                          'MEASURE INITIAL WEIGHT
[illegible]
[illegible]                                          CONVERT VOLUME TO VOLTAGE
[illegible]                                          CALCULATE DESIRED WEIGHT
[illegible]                                          OPEN MEDIUM VALVE
[illegible]                                          MEASURE NEW WEIGHT
[illegible]                                          ASSIGN TO WITH
[illegible]                                          COMPARE WEIGHTS
[illegible]                                          RETURN TO START OF LOOP
[illegible]                                          CLOSE MEDIUM VALVE
[illegible]
[illegible]
[illegible]
[illegible] ON TIMER# 1,3600000 GOTO 780             WAIT 60 MINUTES
[illegible] GOTO [illegible]                         IDLE LOOP
[illegible] OFF TIMER# 1
[illegible]                                          RESET MINIMUM OXYGEN
[illegible]                                          SET COUNTER
[illegible]                                          SET OXYGEN TOTAL
[illegible]                                          INCREMENT COUNTER
[illegible]                                          MEASURE OXYGEN
[illegible]
[illegible]                                          SUM TOTAL
[illegible]                                          TEST COUNTER
[illegible]
[illegible]                                          COMPUTE AVERAGE OXYGEN
[illegible]                                          IF DECREASING, CONTINUE
[illegible]                                          COMPUTE NET INCREASE
[illegible]                                          IF INCREASE SUFFICIENT, CYCLE
[illegible]
[illegible]                                          RESET MINIMUM OXYGEN
[illegible]                                          CONTINUE TO START OF LOOP
[illegible]                                          MEASURE WEIGHT
[illegible]                                          ASSIGN TO WITA
[illegible]                                          CALCULATE DESIRED WEIGHT
[illegible]                                          OPEN HARVESTING VALVE
```

```
590 OUTPUT 7[illegible] ,"D[illegible]"                  [illegible]
600 ENTER 7[illegible] ;WIT
610 IF WIT<>WITD THEN GOTO [illegible]      [illegible]
620 GOTO 590                                [illegible]
630 OUTPUT 7[illegible] ,"[illegible]"      [illegible]
640 WAIT [illegible]
650 OUTPUT 7[illegible] ,"[illegible]"
660 ENTER 7[illegible] ;U[illegible]
670 VOLT=[illegible]
680 OUTPUT 7[illegible] ,"[illegible]"      [illegible]
690 ON TIME [illegible]
700 SET TIME [illegible]
710 WAIT 5000                               [illegible]
720 OUTPUT 709 ;"[illegible]"               [illegible]
    SVALUE=[illegible]                      [illegible]
740 IF SVALUE[illegible] THEN GOTO [illegible]
750 SVAL=[illegible]
760 IF SVAL[illegible] THEN GOTO 810
770 OUTPUT 7[illegible] ,"[illegible]1"     [illegible]
780 WAIT [illegible]                        [illegible]
790 OUTPUT 7[illegible] ,"[illegible]1"     [illegible]
800 GOTO 890
810 IF SVAL[illegible] THEN GOTO [illegible]
820 OUTPUT 7[illegible] ,"[illegible]"      [illegible]
830 WAIT [illegible]                        [illegible]
840 OUTPUT 7[illegible] ,"[illegible]"      [illegible]
850 GOTO 890
860 OUTPUT 7[illegible] ,"[illegible]1"     [illegible]
870 WAIT [illegible]                        [illegible]
880 OUTPUT 7[illegible] ,"[illegible]1"     [illegible]
890 COUNT[illegible]=COUNT[illegible]       [illegible]
900 [illegible]
910 PRINT USING [illegible]                 [illegible] MINUTES
[illegible]
920 IMAGE [illegible]
930 [illegible]
940 GOTO [illegible]                        [illegible]
950 END
```

```
[illegible]                                        MEASURE INITIAL WEIGHT
[illegible]                                        CONVERT VOLUME TO [illegible]
[illegible]                                        CALCULATE DESIRED WEIGHT
[illegible]                                        OPEN MEDIUM VALVE
[illegible]                                        MEASURE NEW WEIGHT
[illegible]                                        ASSIGN TO [illegible]
[illegible]                                        COMPARE WEIGHTS
[illegible]                                        RETURN TO START OF LOOP
[illegible]                                        CLOSE MEDIUM VALVE
[illegible]
[illegible]                                        WAIT [illegible] MINUTES
[illegible]                                        IDLE LOOP
[illegible]
[illegible]                                        RESET MINIMUM OXYGEN
[illegible]                                        SET COUNTER
[illegible]                                        SET OXYGEN TOTAL
[illegible]                                        INCREMENT COUNTER
[illegible]                                        MEASURE OXYGEN
[illegible]
[illegible]                                        ADD TOTAL
[illegible]                                        CHECK COUNTER
[illegible]
[illegible]                                        COMPUTE AVERAGE OXYGEN
[illegible]                                        IF DECREASING, CONTINUE
[illegible]                                        COMPUTE NET INCREASE
[illegible]                                        IF INCREASE SUFFICIENT, CYCLE
[illegible]
[illegible]                                        RESET MINIMUM OXYGEN
[illegible]                                        CONTINUE TO START OF LOOP
[illegible]                                        START STARVATION PERIOD
```

```
590 GOTO 590 !                                         IDLE LOOP
600 OFF TIMER#
610 OUTPUT 709 ;"ICO" !
620 ENTER 709 ; WITO !                                 MEASURE WEIGHT
630 WTID=WITO+(UL-[illegible]OF  0004) !               ASSIGN TO WIT
640 OUTPUT 709 ;"CLS0" !                               CAL [illegible] WEIGHT
650 OUTPUT 709 ;"RCV0" !                               OPEN [illegible] VALVE
660 ENTER 709 ; WIT !                                  MEASURE NEW WEIGHT
670 IF WIT = WTID THEN GOTO 690                        COMPARE WEIGHTS
680 GOTO 650 !                                         RETURN TO [illegible] LOOP
690 OUTPUT [illegible] ;"OPEN[illegible]" !            CLOSE [illegible] VALVE
700 WAIT 5000 !
710 OUTPUT 709 ;"[illegible]"
720 ENTER 709 ; WIF
730 [illegible] WIF-WIT0
740 OUTPUT 709 ;"CLS10" !
750 MTIME=TIME 50                                      OPEN [illegible]
760 SETTIME 0,1
770 WAIT 5 000 !
780 OUTPUT 709 ;"OPEN[illegible]" !                    WAIT ONE MINUTE
790 SVALVE SVALVE+1                                    CLOSE [illegible]
800 IF SVALVE[illegible] THEN GOTO [illegible]         INCREMENT SAMPLE VALVE #
[illegible]
820 IF SVALVE[illegible] THEN GOTO 870
830 OUTPUT 709 ;"CLS1" !                               OPEN SAMPLE VALVE #1
840 WAIT 90000                                         WAIT ONE MINUTE [illegible]
850 OUTPUT 709 ;"OPEN1" !                              CLOSE SAMPLE VALVE #1
860 GOTO 750
870 IF SVALVE=3 THEN GOTO 920
880 OUTPUT 709 ;"CLS20" !                              OPEN SAMPLE VALVE #
890 WAIT 90000 !                                       WAIT ONE MINUTE [illegible]
900 OUTPUT 709 ;"OPN20" !                              CLOSE SAMPLE VALVE #2
910 GOTO 950
920 OUTPUT 709 ;"CLS21" !                              OPEN SAMPLE VALVE #
930 WAIT 90000 !                                       WAIT ONE MINUTE [illegible]
940 OUTPUT 709 ;"OPN21" !                              CLOSE SAMPLE VALVE #
950 CCOUNT=CCOUNT+1 !                                  INCREMENT CYCLE COUNTER
960 DXY=[illegible]
970 PRINT USING 980 ; "CYCLE NUMBER" CCOUNT " COMPLETED AFTER" MTIME," MINUTE.
D.OXY =",DXY[illegible]
980 IMAGE [illegible]
990 BEEP[illegible]
1000 GOTO 590 !                                        CYCLE IS [illegible]
1010 END
```

```
10 REM *** PHASING CONTROL PROGRAM VERSION 6 ***  AUG.  1, 1988
20 VCOR1=0
30 CCOUNT=0
40 VCOR2=0
50 SVALVE=0
60 PRINTER IS 701
70 DISP "ENTER DOSING VOLUME IN ML"
80 INPUT DVOL
90 CVOL=DVOL*.000156  '                          CONVERT VOLUME TO VOLTAGE
100 DISP "ENTER STARVATION PERIOD IN MINUTES"
110 INPUT MSTARVE
120 SSTARVE=MSTARVE*60000+1
130 DISP "BYPASS DOSING? YES=1, NO=0"
140 INPUT BYPASS
150 DISP "ENTER MINIMUM OXYGEN CONC. IN % SAT"
160 INPUT OXYGEN
170 MINOXY=OXYGEN 92.5
180 DISP "ENTER CURRENT TIME"
190 DISP "USING FORMAT :HOURS, MINUTES, SECONDS SINCE MIDNIGHT"
200 INPUT HRS,MINU,SECS
210 A=HRS*3600+MINU*60+SECS
220 DISP "ENTER DATE USING FORMAT MM,DD"
230 INPUT M,D
240 PRINT USING "18A,X,DD,X,3A,X,DD,X,11A,X,DD,9A,X,DD" ; "PHASING STARTED AT",H
RS,"HRS",MINU,"MINS ON DAY",D," OF MONTH",M
250 SETTIME 0,1
260 IF BYPASS=1 THEN GOTO 410
270 OUTPUT 709 ;"DCV1" '                         MEASURE INITIAL WEIGHT
280 ENTER 709 ; WTI
290 CVOL=DVOL*.000156  '                         CONVERT VOLUME TO VOLTAGE
300 WTD=WTI+CVOL+VCOR1-.0008 '                   CALCULATE DESIRED WEIGHT
310 OUTPUT 709 ;"CLS00" '                        OPEN MEDIUM VALVE
320 OUTPUT 709 ;"DCV2" '                         MEASURE NEW WEIGHT
330 ENTER 709 ; WTA '                            ASSIGN TO WTA
340 IF WTA = WTD THEN GOTO 360 '                 COMPARE WEIGHTS
350 GOTO 320 '                                   RETURN TO START OF LOOP
360 OUTPUT 709 ;"OPN00" '                        CLOSE MEDIUM VALVE
370 WAIT 5000
380 OUTPUT 709 ;"DCV2"
390 ENTER 709 ; WTF
400 VCOR1=WTF-WTD
410 ON TIMER# 1,3600000 GOTO 430 '               WAIT 60 MINUTES
420 GOTO 420 '                                   IDLE LOOP
430 OFF TIMER# 1
440 SCOUNT=0 '                                   SET COUNTER
450 OXYTA=0 '                                    SET OXYGEN TOTAL
460 SCOUNT=SCOUNT+1 '                            INCREMENT COUNTER
470 OUTPUT 709 ;"DCV1" '                         MEASURE OXYGEN
480 ENTER 709 ; OXY1
490 OXYTA=OXYTA+OXY1 '                           SUM TOTAL
500 IF SCOUNT=100 THEN GOTO 520 '                CHECK COUNTER
510 GOTO 460
520 OXY1=OXYTA/SCOUNT '                          COMPUTE AVERAGE OXYGEN
530 IF OXY1<MINOXY THEN GOTO 550 '               IF LESS THAN MIN, GO TO 550
540 GOTO 460 '                                   CONTINUE TO START OF LOOP
550 ON TIMER# 2,SSTARVE GOTO 570 '               START STARVATION PERIOD
560 GOTO 560 '                                   IDLE LOOP
570 OFF TIMER# 2
580 OUTPUT 709 ;"DCV2" '                         MEASURE WEIGHT
```

```
590 ENTER 707 : WITA !                                      ASSIGN TO WITA
600 WITD=WITA+CVOL*VCOR2*.0008 !                            CALCULATE DESIRED WEIGHT
610 OUTPUT 709 ;"CLS01" !                                   OPEN HARVESTING VALVE
620 OUTPUT 709 ;"DC003" !                                   MEASURE NEW WEIGHT
630 ENTER 707 : WITA
640 IF WITA>=WITD THEN GOTO 660 !                           COMPARE WEIGHTS
650 GOTO 620 !                                              RESTART HARVESTING LOOP
660 OUTPUT 709 ;"OPN01" !                                   CLOSE HARVESTING VALVE
670 WAIT 5000
680 OUTPUT 709 ;"RL"
690 ENTER 709 : WITF
700 VCOR2=WITF-WITD
710 OUTPUT 709 ;"CLS10" !                                   OPEN DOSING VALVE
720 ON TIME TIME 760
730 SET TIME 0.1
740 WAIT 60000 !                                            WAIT ONE MINUTE
750 OUTPUT 709 ;"OPN10" !                                   CLOSE DOSING VALVE
760 SVALVE=SVALVE+1 !                                       INCREMENT SAMPLE VALVE #
770 IF SVALVE 4 THEN GOTO 790
780 SVALVE=1
790 IF SVALVE 1 THEN GOTO 840
800 OUTPUT 709 ;"CLS11" !                                   OPEN SAMPLE VALVE #1
810 WAIT 90000 !                                            WAIT ONE MIN + 30 SEC
820 OUTPUT 709 ;"OPN11" !                                   CLOSE SAMPLE VALVE #1
830 GOTO 920
840 IF SVALVE=2 THEN GOTO 890
850 OUTPUT 709 ;"CLS20" !                                   OPEN SAMPLE VALVE #2
860 WAIT 90000 !                                            WAIT ONE MIN + 30 SEC
870 OUTPUT 709 ;"OPN20" !                                   CLOSE SAMPLE VALVE #2
880 GOTO 920
890 OUTPUT 709 ;"CLS21" !                                   OPEN SAMPLE VALVE #3
900 WAIT 90000 !                                            WAIT ONE MIN + 30 SEC
910 OUTPUT 709 ;"OPN21" !                                   CLOSE SAMPLE VALVE #3
920 CCOUNT=CCOUNT+1 !                                       INCREMENT CYCLE COUNTER
930 OXY3=OXY1*92.5
940 PRINT USING 950 ; "CYCLE NUMBER",CCOUNT," COMPLETED AFTER",MTIME," MINUTES;
D.OXY =",OXY3," % SAT"
950 IMAGE 13A,DDD,16A,DDDD,17A,DD.D,6A
960 BYPASS=0
970 GOTO 270 !                                              CYCLE IS REPEATED
980 END
```

```
10 REM *** PHASING CONTROL PROGRAM VERSION 7 ***  SEPT 11, 1980
20 VCOR1=0
30 CCOUNT=0
40 VCOR2=0
50 SVALVE=0
55 TEST=0
60 MINOXY=2
70 PRINTER IS 701
80 DISP "ENTER DOSING VOLUME IN ML"
90 INPUT DVOL
100 CVOL=DVOL*.000166 !                              CONVERT VOLUME TO VOLTAGE
110 DISP "ENTER STARVATION PERIOD IN MINUTES"
120 INPUT MSTARVE
130 SSTARVE=MSTARVE*60000+1
140 DISP "BYPASS DOSING? YES=1, NO=0"
150 INPUT BYPASS
160 DISP "ENTER CURRENT TIME"
170 DISP "USING FORMAT :HOURS, MINUTES, SECONDS SINCE MIDNIGHT"
180 INPUT HRS,MINU,SECS
190 A=HRS*3600+MINU*60+SECS
200 DISP "ENTER DATE USING FORMAT MM,DD"
210 INPUT M,D
220 PRINT USING "18A,X,DD,X,3A,X,DD,X,11A,X,DD,9A,X,DD" ; "PHASING STARTED AT",H
RS,"HRS",MINU,"MINS ON DAY",D," OF MONTH",M
230 SETTIME 0,1
240 OUTPUT 709 ;"DCV3"
250 ENTER 709 ; WTIF
260 IF BYPASS=1 THEN GOTO 410
270 OUTPUT 709 ;"DCV3" !                             MEASURE INITIAL WEIGHT
280 ENTER 709 ; WTI
290 CVOL=DVOL*.0001667 !                             CONVERT VOLUME TO VOLTAGE
300 WTTD=WTI+CVOL-VCOR1-.0008 !                      CALCULATE DESIRED WEIGHT
310 OUTPUT 709 ;"CLS00" !                            OPEN MEDIUM VALVE
320 OUTPUT 709 ;"DCV3" !                             MEASURE NEW WEIGHT
330 ENTER 709 ; WTTA !                               ASSIGN TO WTTA
340 IF WTTA = WTTD THEN GOTO 360 !                   COMPARE WEIGHTS
350 GOTO 320 !                                       RETURN TO START OF LOOP
360 OUTPUT 709 ;"OPN00" !                            CLOSE MEDIUM VALVE
370 WAIT 5000
380 OUTPUT 709 ;"DCV3"
390 ENTER 709 ; WTTF
400 VCOR1=WTTF-WTTD
410 ON TIMER# 1,3600000 GOTO 430 !                   WAIT 60 MINUTES
420 GOTO 420 !                                       IDLE LOOP
430 OFF TIMER# 1
440 MINOXY=2 !                                       RESET MINIMUM OXYGEN
450 SCOUNT=0 !                                       SET COUNTER
460 OXY1A=0 !                                        SET OXYGEN TOTAL
470 SCOUNT=SCOUNT+1 !                                INCREMENT COUNTER
480 OUTPUT 709 ;"DCV1" !                             MEASURE OXYGEN
490 ENTER 709 ; OXY1
500 OXY1A=OXY1A+OXY1 !                               SUM TOTAL
510 IF SCOUNT=100 THEN GOTO 530 !                    CHECK COUNTER
520 GOTO 470
530 OXY1=OXY1A/SCOUNT !                              COMPUTE AVERAGE OXYGEN
540 IF OXY1<MINOXY THEN GOTO 580 !                   IF DECREASING, CONTINUE
550 DELTAOXY=OXY1-MINOXY !                           COMPUTE NET INCREASE
560 IF DELTAOXY>.0008 THEN GOTO 595 !                IF INCREASE SUFFICIENT, CYCLE
570 GOTO 150
```

```
580 MINOXY=OXY1 !                                         RESET MINIMUM OXYGEN
585 TEST=TEST+1
590 GOTO 450 !                                            CONTINUE TO START OF LOOP
595 IF TEST<2 THEN GOTO 450
600 ON TIMER# 2,SSTARVE GOTO 620 !                        START STARVATION PERIOD
610 GOTO 610 !                                            IDLE LOOP
620 OFF TIMER# 2
630 OUTPUT 709 ;"DCV03" !                                 MEASURE WEIGHT
640 ENTER 709 ; WTI !                                     ASSIGN TO WTIA
650 WTEN=WTIA-WTF
660 WTID=WTF+(VOL-VLOR)*.0008 !                 CALCULATE DESIRED WEIGHT
670 WTD=WTID*6000
680 OUTPUT 709 ;"CLS01" !                                 OPEN HARVESTING VALVE
690 OUTPUT 709 ;"DCV03" !                                 MEASURE NEW WEIGHT
700 ENTER 709 ; WTA
710 IF WTA = WTD THEN GOTO 730 !                          COMPARE WEIGHTS
720 GOTO 690 !                                            RESTART HARVESTING LOOP
730 OUTPUT 709 ;"OPN01" !                                 CLOSE HARVESTING VALVE
740 WAIT 5000
750 OUTPUT 709 ;"DCV03"
760 ENTER 709 ; WTF
770 VCOL=WTF-WTD
780 OUTPUT 709 ;"CLS10" !                                 OPEN DOSING VALVE
785 OUTPUT 709 ;"CLS11"
790 HTIME=TIME/60
800 SETTIME 0,1
810 WAIT 300000 !                                         WAIT FIVE MINUTES
820 OUTPUT 709 ;"OPN10" !                                 CLOSE DOSING VALVE
825 OUTPUT 709 ;"OPN11"
830 GOTO 990
835 SVALVE=SVALVE+1 !                                     INCREMENT SAMPLE VALVE #
840 IF SVALVE<4 THEN GOTO 860
850 SVALVE=1
860 IF SVALVE<>1 THEN GOTO 910
870 OUTPUT 709 ;"CLS11" !                                 OPEN SAMPLE VALVE #1
880 WAIT 90000 !                                          WAIT ONE MIN + 30 SEC
890 OUTPUT 709 ;"OPN11" !                                 CLOSE SAMPLE VALVE #1
900 GOTO 990
910 IF SVALVE=3 THEN GOTO 960
920 OUTPUT 709 ;"CLS20" !                                 OPEN SAMPLE VALVE #2
930 WAIT 120000 !                                         WAIT ONE MIN + 30 SEC
940 OUTPUT 709 ;"OPN20" !                                 CLOSE SAMPLE VALVE #2
950 GOTO 990
960 OUTPUT 709 ;"CLS21" !                                 OPEN SAMPLE VALVE #3
970 WAIT 120000 !                                         WAIT ONE MIN + 30 SEC
980 OUTPUT 709 ;"OPN21" !                                 CLOSE SAMPLE VALVE #3
990 CCOUNT=CCOUNT+1 !                                     INCREMENT CYCLE COUNTER
1000 OXY=MINOXY*92.5
1010 PRINT USING 1020 ; "CYCLE ",CCOUNT," COMPLETED IN",HTIME," MIN: D.OXY =",OX
Y," % SAT",WTF," MG LOAD"
1020 IMAGE 6A,DDD,10A,DDDD,13A,DD.D,6A.DDDD.4A
1030 BYPASS 0
1040 GOTO 200 !                                           CYCLE IS REPEATED
1050 END
```

```
10 REM *** PHASING CONTROL PROGRAMS VERSION 3 ***  OCT. 27, 1988
20 VCOR1=0
30 CCOUNT=0
40 VCOR2=0
50 SVALVE=0
55 TEST=0
60 MINOXY=2
70 PRINTER IS 701
80 DISP "ENTER DOSING VOLUME IN ML"
90 INPUT DVOL
100 CVOL=DVOL*.000165  '                          CONVERT VOLUME TO VOLTAGE
110 DISP "ENTER STARVATION PERIOD IN MINUTES"
120 INPUT MSTARVE
125 DISP "ENTER MAX CYCLE TIME IN MINUTES"
127 INPUT STIME
130 SSTARVE=MSTARVE*60000+1
160 DISP "ENTER CURRENT TIME"
170 DISP "USING FORMAT :HOURS, MINUTES, SECONDS SINCE MIDNIGHT"
180 INPUT HRS,MINU,SECS
190 A=HRS*3600+MINU*60+SECS
200 DISP "ENTER DATE USING FORMAT MM,DD"
210 INPUT M,D
220 PRINT USING "18A,X,DD,X,3A,X,DD,X,11A,X,DD,9A,X,DD" : "PHASING STARTED AT ",H
RS,"HRS",MINU,"MINS ON DAY",D," OF MONTH",M
230 SETTIME 0,1
240 OUTPUT 709 ;"DCV3"
250 ENTER 709 ; WTTF
260 GOTO 410
270 OUTPUT 709 ;"DCV3" '                        MEASURE INITIAL WEIGHT
280 ENTER 709 ; WTI
290 CVOL=DVOL*.0001657 '                        CONVERT VOLUME TO VOLTAGE
300 WTTD=WTI+CVOL-VCOR1-.0008 '                 CALCULATE DESIRED WEIGHT
305 IF TIME<STIME THEN GOTO 310
307 OUTPUT 709 ;"CLS21"
308 GOTO 320
310 OUTPUT 709 ;"CLS00" '                       OPEN MEDIUM VALVE
320 OUTPUT 709 ;"DCV3" '                        MEASURE NEW WEIGHT
330 ENTER 709 ; WTTA '                          ASSIGN TO WTTA
340 IF WTTA = WTTD THEN GOTO 360 '              COMPARE WEIGHTS
350 GOTO 320 '                                  RETURN TO START OF LOOP
360 OUTPUT 709 ;"OPN00" '                       CLOSE MEDIUM VALVE
365 OUTPUT 709 ;"OPN21"
370 WAIT 5000
380 OUTPUT 709 ;"DCV3"
390 ENTER 709 ; WTTF
400 VCOR1=WTTF-WTTD
405 GOTO 780
410 ON TIMER# 1,3600000 GOTO 430 '              WAIT 60 MINUTES
420 GOTO 420 '                                  IDLE LOOP
430 OFF TIMER# 1
440 MINOXY=2 '                                  RESET MINIMUM OXYGEN
450 SCOUNT=0 '                                  SET COUNTER
460 OXY1A=0 '                                   SET OXYGEN TOTAL
470 SCOUNT=SCOUNT+1 '                           INCREMENT COUNTER
480 OUTPUT 709 ;"DCV13" '                       MEASURE OXYGEN
490 ENTER 709 ; OXY1
500 OXY1A=OXY1A+OXY1 '                          SUM TOTAL
510 IF SCOUNT=100 THEN GOTO 530 '               CHECK COUNTER
520 GOTO 470
```

```
500 OXY1=OXY1/SCOUNT !                              COMPUTE AVERAGE OXYGEN
510 IF OXY1 MINOX  THEN GOTO 590 !                  IF DECREASING, CONTINUE
520 DELTAOX=(OXY1-MINOX) !                          COMPUTE NEW INCREASE
530 IF DELTAOX . 004 THEN GOTO 580 !                IF INCREASE SUFFICIENT  CYCLE

540 GOTO 450
550 MINOX=OXY1 !                                    RESET MINIMUM OXYGEN
560 TEST=TEST+1
570 GOTO 450 !                                      CONTINUE TO START OF LOOP
580 IF TEST   THEN GOTO 450
600 ON TIMER# 2,SSTARVE GOTO 620 !                  START STARVATION PERIOD
610 GOTO 610 !                                      IDLE LOOP
620 OFF TIMER# 2
630 OUTPUT 709 ;"DC??" !                            MEASURE WEIGHT
640 ENTER 709 ; WTA !                               ASSIGN TO WTA
650 WTFM=WTA-WTF
660 WTTD=WTF+CVOL*CONC+.0006 !              CALCULATE DESIRED WEIGHT
670 WT??=WTFM*6000
680 OUTPUT 709 ;"CLS01" !                           OPEN HARVESTING VALVE
690 OUTPUT 709 ;"DC?0" !                            MEASURE NEW WEIGHT
700 ENTER 709 ; WTH
710 IF WTH - WTD THEN GOTO 730 !                    COMPARE WEIGHTS
720 GOTO 690 !                                      RESTART HARVESTING LOOP
730 OUTPUT 709 ;"OPN01"                             CLOSE HARVESTING VALVE
740 WAIT 1000
750 OUTPUT 709 ;"DC??"
760 ENTER 709 ; WTF
770 VOL=WTF-WTD
780 HTIME=TIME/60
790 GOTO 870
800 OUTPUT 709 ;"CLS10" !                           OPEN DOSING VALVE
805 OUTPUT 709 ;"CLS11"
806 MTIME=TIME/60
808 SETTIME 0,1
810 WAIT 60000 !                                    WAIT FULL MINUTES
820 OUTPUT 709 ;"OPN10" !                           CLOSE DOSING VALVE
830 OUTPUT 709 ;"OPN11"
832 OUTPUT 709 ;"DC?0"
834 ENTER 709 ; WTTF
835 GOTO 990
840 SVALVE=SVALVE+1 !                               INCREMENT SAMPLE VALVE #
850 IF SVALVE 3 THEN GOTO 860
855 SVALVE=1
860 IF SVALVE 1 THEN GOTO 910
870 OUTPUT 709 ;"CLS11" !                           OPEN SAMPLE VALVE #1
880 WAIT 70000
885 OUTPUT 709 ;"OPN10"
890 OUTPUT 709 ;"OPN11" !                           CLOSE SAMPLE VALVE #1
900 GOTO 990
910 IF SVALVE   THEN GOTO 960
920 OUTPUT 709 ;"CLS20" !                           OPEN SAMPLE VALVE #
930 WAIT 30000
935 OUTPUT 709 ;"OPN10"
940 OUTPUT 709 ;"OPN20" !                           CLOSE SAMPLE VALVE #2
950 GOTO 990
960 OUTPUT 709 ;"CLS21" !                           OPEN SAMPLE VALVE #2
970 WAIT 120000 !                                   WAIT ONE MIN + 30 SEC
980 OUTPUT 709 ;"OPN21" !                           CLOSE SAMPLE VALVE #2
990 CCOUNT=CCOUNT+1 !                               INCREMENT CYCLE COUNTER
1000 OXY=(MINOX)*2.5
1010 PRINT USING 10,0 ; "CYCLE ",CCOUNT," COMPLETED IN",MTIME," MIN: D.OXY =",OX
```

```
Y3," % SAT",QTFY," IN FORM"
1020 IMAGE 6A,DDD,13X,DDDD,1X,A,DD.D,6X,DDDD,3A
1030 B:PASS=0
1040 GOTO 410 !                                 CYCLE IS REPEATED
1050 END
```

```
10 REM *** FEEDING CONTROL PROGRAM VERSION [illegible] *** DEC . 2[illegible] 1988
20 VCOR1=0
30 SCOUNT=0
35 FCOUNT=0
40 VCOR=0
45 ICOUNT=0
50 SWEIGHT=0
55 TEST=0
60 MINUX=-
70 PRINTER IS 701
80 DISP "ENTER DOSING VOLUME IN ML"
90 INPUT DVOL
100 VOL=DVOL*.001667 !                          CONVERT VOLUME TO VOLTAGE
110 DISP "ENTER STARVATION PERIOD IN MINUTES"
120 INPUT MSTARVE
130 DISP "ENTER FIRST HARMONIC ORDER AS AN INTEGER
140 INPUT FECOUNT
150 DISP "ENTER SECOND HARMONIC ORDER AS AN INTEGER
160 DISP "NOTE: CANNOT BE AN EVEN MULTIPLE OF FIRST HARMONIC
170 INPUT NNCOUNT
180 [illegible] MSTARVE*60*1000*1
190 [illegible]
200 DISP "[illegible] FORMAT :HOURS, MINUTES, SECONDS, START WEIGHT"
210 INPUT [illegible]
    [illegible]
  [illegible]
240 INPUT M,[illegible]
250 PRINT USING [illegible] ; "FEEDING STARTED AT",H
  ,"HOURS ON DAY",D," OF MONTH",M
  PRINT USING [illegible] : "FIRST HARMONIC IS",FECOUNT,";SECOND HAR
MONIC IS",NNCOUNT
  SETTIME [illegible]
 10 OUTPUT 709 ; RC[illegible]"
 50 ENTER 709 ; WIN
  GOTO 310
 70 OUTPUT 709 ;"DC[illegible]" !                MEASURE INITIAL WEIGHT
 80 ENTER 709 ; WIN

 VVOL=DVOL*.001667 !                            CONVERT VOLUME TO VOLTAGE
 00 WIND=WIN+(VOL-VCOR1-.0008) !                CALCULATE DESIRED WEIGHT
 02 IF FCOUNT=FECOUNT THEN GOTO 310 !           CHECK FIRST NUTRIENT COUNTER
 05 IF NCOUNT=NNCOUNT THEN GOTO 1[illegible] !  CHECK SECOND NUTRIENT COUNTER
 07 OUTPUT 709 ;"CL 20" !                       DOSE WITH MAIN MEDIUM
 08 GOTO [illegible]

 10 OUTPUT 709 ;"CL 200" !                      DOSE WITH FIRST NUTRIENT
 11 FCOUNT=0 !                                  RESET COUNTER
  GOTO 20

 1 OUTPUT 709 ;"CLS 1" !                        DOSE WITH SECOND NUTRIENT
 1 NCOUNT=0                                     RESET COUNTER
  OUTPUT 709 ;"[illegible]" !                   MEASURE NEW WEIGHT
   ENTER 709 ; WIN                              ASSIGN TO WIN
 IF WIN [illegible] WIND THEN GOTO [illegible]  COMPARE WEIGHTS
  GOTO [illegible]                              RETURN TO START OF LOOP
 OUTPUT 709 ;"[illegible]"                      CLOSE MEDIUM VALVE
  OUTPUT 709 ;"OPN2" !                          CLOSE MEDIUM VALVE
  OUTPUT 709 ;"OPN21" !                         CLOSE MEDIUM VALVE
  WAIT 3000
 90 OUTPUT 709 ;"[illegible]"
 9 ENTER 709 ; WIN
 00 VCOR1=WIN-WIND !                            CALCULATE CORRECTION VOLUME
```

```
405 GOTO 80
410 ON TIMER# 1,7600000 GOTO 430 '                 WAIT 60 MINUTES
420 GOTO 420 '                                     IDLE LOOP
430 OFF TIMER# 1
440 MINOXY=2 '                                     RESET MINIMUM OXYGEN
450 SCOUNT=0 '                                     SET COUNTER
460 OXYTA=0 '                                      SET OXYGEN TOTAL
470 SCOUNT=SCOUNT+1 '                              INCREMENT COUNTER
480 OUTPUT 709 ;"DCV13" '                          MEASURE OXYGEN
490 ENTER 709 ; OXY1
500 OXYTA=OXYTA+OXY1 '                             SUM TOTAL
510 IF SCOUNT=100 THEN GOTO 530 '                  CHECK COUNTER
520 GOTO 470
530 OXY1=OXYTA/SCOUNT '                            COMPUTE AVERAGE OXYGEN
540 IF OXY1<MINOXY THEN GOTO 580 '                 IF DECREASING, CONTINUE
550 DELTAOXY=OXY1-MINOXY '                         COMPUTE NET INCREASE
560 IF DELTAOXY>.004 THEN GOTO 595 '               IF INCREASE SUFFICIENT, CYCLE
570 GOTO 450
580 MINOXY=OXY1 '                                  RESET MINIMUM OXYGEN
585 TEST=TEST+1
590 GOTO 450 '                                     CONTINUE TO START OF LOOP
595 IF TEST<2 THEN GOTO 450
600 ON TIMER# 2,STARVE GOTO 620 '                  START STARVATION PERIOD
610 GOTO 610 '                                     IDLE LOOP
620 OFF TIMER# 2
630 OUTPUT 709 ;"DCV3" '                           MEASURE WEIGHT
640 ENTER 709 ; WTTA '                             ASSIGN TO WTTA
650 WTEM=WTTA-WTTF
660 WTTD=WTTF+CVOL-VCOR2+.0008 '                   CALCULATE DESIRED WEIGHT
670 WTEV=WTEM*6000
680 OUTPUT 709 ;"CLS01" '                          OPEN HARVESTING VALVE
690 OUTPUT 709 ;"DCV0 " '                          MEASURE NEW WEIGHT
700 ENTER 709 ; WTTA
710 IF WTTA<=WTTD THEN GOTO 730 '                  COMPARE WEIGHTS
720 GOTO 690 '                                     RESTART HARVESTING LOOP
730 OUTPUT 709 ;"OPN01" '                          CLOSE HARVESTING VALVE
740 WAIT 5000
750 OUTPUT 709 ;"DCV3"
760 ENTER 709 ; WTTF
770 VCOR2=WTTF-WTTD '                              CALCULATE CORRECTION VOLUME
772 FCOUNT=FCOUNT+1 '                              INCREMENT COUNTER
774 TCOUNT=TCOUNT+1 '                              INCREMENT COUNTER
775 GOTO 270
780 OUTPUT 709 ;"CLS10" '                          OPEN DOSING VALVE
785 OUTPUT 709 ;"CLS11" '                          OPEN SAMPLE VALVE
790 MTIME=TIME/60 '                                CALCULATE CYCLE TIME
800 SET TIME 0
810 WAIT 300000 '                                  WAIT FIVE MINUTES
820 OUTPUT 709 ;"OPN10" '                          CLOSE DOSING VALVE
822 OUTPUT 709 ;"OPN11" '                          CLOSE SAMPLE VALVE
823 OUTPUT 709 ;"DCV03"
824 ENTER 709 ; WTTF
825 GOTO 990
990 CCOUNT=CCOUNT+1 '                              INCREMENT CYCLE COUNTER
1000 OXY2=MINOXY*92.
1010 PRINT USING 1020 ; "CYCLE ",CCOUNT," COMPLETED IN ",MTIME," MIN DO% ",OX
Y2," % SAT",WTF," ML FOAM"
1020 IMAGE 5A,DDD,12A,DDDD,13A,DD.D,6A,DDDD,3A
1030 BYPASS=0
1040 GOTO 410 '                                    CYCLE IS REPEATED
```

[illegible]

```
10 REM *** PHASING CONTROL PROGRAMME VERSION 10 ***  OCT  21, 1986
20 VCOR1=0
30 CCOUNT=0
40 NCOUNT1=0
50 VCOR2=0
60 NCOUNT2=0
70 SVALVE=0
80 TEST=0
90 MINOX=2
100 PRINTER IS 701
110 DISP "ENTER TOTAL DOSING VOLUME IN ML"
120 INPUT DVOLT
130 DVOLTV=DVOLT*.000166 !                            CONVERT VOLUME TO VOLTAGE
135 DISP "ENTER STARVATION PERIOD IN MINUTES"
136 INPUT MSTARVE
137 SSTARVE=MSTARVE*60000+1
140 DISP "ENTER CONC. OF NUTRIENT #1 IN MOLES/L"
150 INPUT ICONC1
160 DISP "ENTER CONC. OF NUTRIENT #2 IN MOLES/L"
170 INPUT ICONC2
180 DISP "ENTER DESIRED DOSING CONC. OF NUTRIENT #1 [illegible] 50% OF [illegible]"
190 INPUT FCONC1
200 DISP "ENTER DESIRED DOSING CONC. OF NUTRIENT #2 [illegible]"
210 INPUT FCONC2
220 DISP "ENTER DOSING FREQUENCY FOR NUTRIENT #1 (FIRST HARMONIC)"
230 INPUT COUNT1
240 DISP "ENTER DOSING FREQUENCY FOR NUTRIENT #2 (SECOND HARMONIC)"
250 INPUT COUNT2
260 DISP "ENTER CURRENT TIME"
270 DISP "USING FORMAT :HOURS, MINUTES, SECONDS [illegible]"
280 INPUT HRS,MINU,SECS
290 A=HRS*3600+MINU*60+SECS
300 DISP "ENTER DATE USING FORMAT MM.DD"
310 INPUT M,D
320 PRINT USING "18A,X,DD,X,3A,X,DD,X,11A,X,DD [illegible]" ; "PHASING STARTED AT ",H
RS,"HRS",MINU,"MINS ON DAY",D," OF MONTH",M
330 PRINT USING "17A,X,DD,X,17A,X,DD" ; "FIRST HARMONIC IS ",COUNT1,"SECOND HARM
ONIC IS",COUNT2
340 SETTIME 0,1
350 OUTPUT 709 ;"LC0 "
360 ENTER 709 ; WTI
370 GOTO 380
380 OUTPUT 709 ;"TC "
390 ENTER 709 ; WTI
400 DVOL1=FCONC1/ICONC1*DVOLT !                       CALCULATE #1 NUTRIENT VOL.
410 IF NCOUNT1=COUNT1 THEN GOTO 440 !                 CHECK CYCLE COUNTER
420 DVOL1=0
430 GOTO 520
440 NCOUNT1=0
450 WTID=WTI+DVOL1 !                                  CALCULATE DESIRED WEIGHT
460 OUTPUT 709 ;"CL500" !                             [illegible]
470 OUTPUT 709 ;"DO1"
480 ENTER 709 ; WTIA
490 IF WTIA = WTID THEN GOTO 510
500 GOTO 470
510 OUTPUT 709 ;"OPEN00" !                            CLOSE [illegible]
515 WAIT 5000 !                                       PAUSE 5 SECONDS
520 OUTPUT 709 ;"PC"
522 ENTER 709 ; WTIA
```

```
525 DVOL2=FLOAT(IONCL*DELT) !                 CALCULATE #2 NUTRIENT VOL.
530 IF NCOUNT2=COUNT2 THEN GOTO 560 !          CHECK COUNTER
540 DVOL2=0
550 GOTO 570
560 NCOUNT2=0
570 WTTD=WTTD+DVOL2 !                          CALCULATE DESIRED WEIGHT
580 OUTPUT 709 ;"CLS21" !                      OPEN MEDIUM VALVE #21 (2ND)
590 OUTPUT 709 ;"DCV2"
600 ENTER 709 ; WTTF
[illegible] IF WTTF = WTTD THEN GOTO 620
6[illegible]0 GOTO 590
620 OUTPUT 709 ;"OPN21" !                      CLOSE MEDIUM VALVE #21
625 WAIT 5000 !                                PAUSE 5 SECONDS
640 OUTPUT 709 ;"DCV2"
645 ENTER 709 ; WTTF
615 DVOL3=DVOLT-DVOL1-DVOL2 !                  CALCULATE ADDITIONAL VOLUME
650 WTTD=WTTD+DVOL3-VCORR !                    CALCULATE ADDITIONAL WEIGHT
660 IF WTTD = 0 THEN GOTO 770
670 OUTPUT 709 ;"CLS20" !                      OPEN MEDIUM VALVE #20 (3RD)
680 OUTPUT 709 ;"DCV2"
690 ENTER 709 ; WTTF
700 IF WTTF - WTTD THEN GOTO 720
710 GOTO 680
720 OUTPUT 709 ;"OPN20" !                      CLOSE MEDIUM VALVE #20
730 WAIT 5000
740 OUTPUT 709 ;"DCV2"
750 ENTER 709 ; WTTF
760 VCORR=WTTF-WTTD !                          CALCULATE CORRECTION VOLUME
770 GOTO 1[illegible]
780 ON TIMER# 1,1800000 GOTO 800 !             WAIT 30 MINUTES
790 GOTO 790 !                                 IDLE LOOP
800 OFF TIMER# 1
810 [illegible] !                              RESET MINIMUM OXYGEN
820 SE[illegible] !                            SET COUNTER
830 [illegible] !                              SET OXYGEN TOTAL
840 SCOUNT=SCOUNT+1 !                          INCREMENT COUNTER
850 OUTPUT 709 ;"DCV1" !                       MEASURE OXYGEN
860 ENTER 709 ; OXY1
870 OXY1A=OXY1A+OXY1 !                         SUM TOTAL
880 IF SCOUNT=100 THEN GOTO 900 !              CHECK COUNTER
890 GOTO 840
900 OXY1=OXY1A/SCOUNT !                        COMPUTE AVERAGE OXYGEN
910 IF OXY1 MINOXY THEN GOTO 950 !             IF DECREASING, CONTINUE
920 DELTAOXY=OXY1-MINOXY !                     COMPUTE NET INCREASE
930 IF DELTAOXY .012 THEN GOTO 975 !           IF INCREASE SUFFICIENT, CYCLE
940 GOTO 820
950 MINOXY=OXY1 !                              RESET MINIMUM OXYGEN
960 TEST=TEST+1
970 GOTO 820 !                                 CONTINUE TO START OF LOOP
[illegible] MINOXY=OXY1
[illegible] IF TEST [illegible] THEN GOTO [illegible]
[illegible] ON TIMER# [illegible] GOTO 1010           START STARVATION PERIOD
1000 GOTO 1000 !                                IDLE LOOP
1010 OFF TIMER# 2
1020 OUTPUT [illegible] !                       MEASURE WEIGHT
1030 ENTER 709 [illegible] !                    ASSIGN TO WTTH
1040 WTTH=WTTA WTTF
1050 WTTD=WTTH+[illegible]*[illegible]03          CALCULATE DESIRED WEIGHT
1060 OFF [illegible] WTFH+6[illegible]
10[illegible] OUTPUT [illegible] [illegible]1 OPEN HARVESTING VALVE
```

```
1080 OUTPUT 709 :"DCV[illegible]" !                       MEASURE NEW WEIGHT
1090 ENTER 709 : WITB
1100 IF WITA = WITB THEN GOTO 1120 !                        COMPARE WEIGHTS
1110 GOTO 1090 !                                            RESTART HARVESTING LOOP
1120 OUTPUT 709 :"OPEN1" !                                 CLOSE HARVESTING VALVE
1130 WAIT 5000
1140 OUTPUT 709 :"DC[illegible]"
1150 ENTER 709 : WITD
1160 !CORR=WITB-WITD !                                     CALCULATE CORRECTION VOLUME
1170 NCOUNT1=NCOUNT1+1
1180 NCOUNT2=NCOUNT2+1
1190 GOTO 780
1200 OUTPUT 709 :"CLS10" !                                 OPEN DOSING VALVE
1210 OUTPUT 709 :"CLS11" !                                 OPEN SAMPLE VALVE
1220 MTIME=TIME/60 !                                       CALCULATE CYCLE TIME
1230 SETTIME 0.1 !                                         RESET CLOCK
1240 WAIT 300000 !                                         WAIT FIVE MINUTES
1250 OUTPUT 709 :"OPN10" !                                 CLOSE DOSING VALVE
1260 OUTPUT 709 :"OPN11" !                                 CLOSE SAMPLE VALVE
1270 OUTPUT 709 :"DCV[illegible]"
1280 ENTER 709 : WITF
1290 CCOUNT=CCOUNT+1 !                                     INCREMENT CYCLE COUNTER
1300 DAY=MTIME/[illegible]
1310 PRINT USING 1320 : "CYCLE ",CCOUNT," COMPLETED IN",MTIME," MIN  D.DY  [illegible]
[illegible] " [illegible]T",WITF," ML FORM"
1320 IMAGE 6A,DDD,13A,DDDD,13A,DD.D,6A,DDDD,3A
1330 BYPASS=0
1340 GOTO 780 !                                            CYCLE IS FINISHED
1350 END
```

```
10 REM *** PHASING CONTROL PROGRAM VERSION II *** NOV 14, 1989
20 VCONC1=0
30 CCOUNT=0
35 COUNT1=1
40 NCOUNT1=0
50 VCONC2=0
60 NCOUNT2=0
70 SVALVE=0
80 TEST=0
90 [illegible]
100 PRINTER IS 701
110 DISP "ENTER TOTAL DOSING VOLUME IN ML"
120 INPUT DVOLT
130 DVOLTV=DVOLT*.0001007 !                           CONVERT VOLUME TO VOLTAGE
135 DISP "ENTER STARVATION PERIOD IN MINUTES"
140 INPUT MSTARVE
145 SSTARVE=MSTARVE*60000+1
150 DISP "ENTER CONC. OF NUTRIENT #1 IN MOLES L"
155 INPUT NCONC1
160 DISP "ENTER CONC. OF NUTRIENT #2 IN MOLES L"
170 INPUT NCONC2
180 DISP "ENTER DESIRED DOSING CONC. OF NUTRIENT #1 (MAX 50% OF ORIGINAL "
190 INPUT DCONC1
200 DISP "ENTER MAX. CONCENTRATION OF NUTRIENT #2 (MAX 50% OF ORIGINAL)"
210 INPUT MAXCON2
220 DISP "ENTER MIN. CONCENTRATION OF NUTRIENT #2 "
230 INPUT MINCON2
240 DISP "ENTER CYCLE PERIOD IN NO. OF CELL GENERATIONS"
250 INPUT PERIOD
255 PERIOD=PERIOD/2
260 DISP "ENTER CURRENT TIME"
270 DISP "USING FORMAT :HOURS. MINUTES. SECONDS SINCE MIDNIGHT"
280 INPUT HRS,MINU,SECS
290 T=HRS*3600+MINU*60+SECS
300 DISP "ENTER DATE USING FORMAT MM.DD"
310 INPUT M,D
320 PRINT USING "16A,X,DD,X,3A,X,DD,X,11A,X,DD,9A,X,DD" ; "PHASING STARTED AT",H
RS,"HRS",MINU,"MINS ON DAY",D," OF MONTH",M
330 PRINT USING "21A,X,DD,X,21A,X,D.DDE,X,10A,X,D.DDE,X,5A" ; "SQUARE WAVE PERIO
D IS",PERIOD,"CELL CYCLES: MAX CONC",MAXCON2,": MIN CONC",MINCON2,"MOLAR"
340 SETTIME 0,1
350 OUTPUT 709 ;"DCV2"
360 ENTER 709 ; WTF
370 GOTO 390
380 OUTPUT 709 ;"DCV2"
390 ENTER 709 ; WTI
400 DVOL1=DCONC1/NCONC1*DVOLTV !                      CALCULATE #1 NUTRIENT VOL.
410 IF NCOUNT1=COUNT1 THEN GOTO 440 !                 CHECK CYCLE COUNTER
420 DVOL1=0
430 GOTO 530
440 NCOUNT1=0
450 WTID=WTI+DVOL1 !                                  CALCULATE DESIRED WEIGHT
460 OUTPUT 709 ;"CLS00" !                             OPEN MEDIUM VALVE #00 (FIRST)
470 OUTPUT 709 ;"DCV2"
480 ENTER 709 ; WTIA
490 IF WTIA >= WTID THEN GOTO 510
500 GOTO 470
510 OUTPUT 709 ;"OPN00" !                             CLOSE MEDIUM VALVE #00
520 WAIT 5000 !                                       PAUSE 5 SECONDS
```

```
510 OUTPUT 709 :"DCV3"
520 ENTER 709 : WTTA
522 IF NCOUNT2=PERIOD2 THEN NCOUNT2=0
525 IF NCOUNT2 = PERIOD2 THEN GOTO 540 !                  CHECK COUNTER
530 DVOL2=MINCON2 ICONC2*DVOLTV !                        CALCULATE DOSING VOLUME
535 GOTO 570
540 DVOL2=MAXCON2 ICONC2*DVOLTV
570 WTTD=WTTA+DVOL2 !                                    CALCULATE DESIRED WEIGHT
580 OUTPUT 709 :"CLS21" !                                OPEN MEDIUM VALVE #21 (CH0)
590 OUTPUT 709 :"DCV3"
600 ENTER 709 : WTTB
610 IF WTTB = WTTD THEN GOTO 630
620 GOTO 590
630 OUTPUT 709 :"OPN21" !                                CLOSE MEDIUM VALVE #21
635 WAIT 5000 !                                          PAUSE 5 SECONDS
640 OUTPUT 709 :"DCV3"
642 ENTER 709 : WTTC
645 DVOL3=DVOLTV-DVOL1-DVOL2 !                           CALCULATE ADDITIONAL VOLUME
650 WTTD=WTTC+DVOL3-VCOR1 !                              CALCULATE ADDITIONAL WEIGHT
660 IF WTTD = 0 THEN GOTO 720
670 OUTPUT 709 :"CLS20" !                                OPEN MEDIUM VALVE #20 (CH0)
680 OUTPUT 709 :"DCV3"
690 ENTER 709 : WTTF
700 IF WTTF = WTTD THEN GOTO 720
710 GOTO 680
720 OUTPUT 709 :"OPN20" !                                CLOSE MEDIUM VALVE #20
730 WAIT 5000
740 OUTPUT 709 :"DCV3"
750 ENTER 709 : WTTF
760 VCOR1=WTTF-WTTD !                                    CALCULATE CORRECTION VOLUME
770 GOTO 1200
780 ON TIMER# 1,2400000 GOTO 800 !                       WAIT 40 MINUTES
790 GOTO 790 !                                           IDLE LOOP
800 OFF TIMER# 1
810 MINOXY=2 !                                           RESET MINIMUM OXYGEN
820 SCOUNT=0 !                                           SET COUNTER
830 OXY1A=0 !                                            SET OXYGEN TOTAL
840 SCOUNT=SCOUNT+1 !                                    INCREMENT COUNTER
850 OUTPUT 709 :"DCV13" !                                MEASURE OXYGEN
860 ENTER 709 : OXY1
870 OXY1A=OXY1A+OXY1 !                                   SUM TOTAL
880 IF SCOUNT=200 THEN GOTO 900 !                        CHECK COUNTER
890 GOTO 840
900 OXY1=OXY1A/SCOUNT !                                  COMPUTE AVERAGE OXYGEN
910 IF OXY1<MINOXY THEN GOTO 950 !                       IF DECREASING, CONTINUE
920 DELTAOXY=OXY1-MINOXY !                               COMPUTE NET INCREASE
930 IF DELTAOXY>.012 THEN GOTO 975 !                     IF INCREASE SUFFICIENT, FEED
940 GOTO 820
950 MINOXY=OXY1 !                                        RESET MINIMUM OXYGEN
960 TEST=TEST+1
970 GOTO 820 !                                           CONTINUE TO START OF LOOP
975 MINOXY=OXY1
980 IF TEST<2 THEN GOTO 820
990 ON TIMER# 2,STARVE GOTO 1010 !                        START STARVATION PERIOD
1000 GOTO 1000 !                                           IDLE LOOP
1010 OFF TIMER# 2
1020 OUTPUT 709 :"DCV3" !                                  MEASURE WEIGHT
1030 ENTER 709 : WTTA !                                    ASSIGN TO WTTA
1040 WTFM=WTTA-WTTF
1050 WTTD=WTTF+DVOLTV-VCOR2+.0000 !                          CALCULATE DESIRED WEIGHT
```

```
1060 WTFV=WTF1+5000
1070 OUTPUT 709 :"CLS01" !                                   OPEN HARVESTING VALVE
1080 OUTPUT 709 :"DCV02" !                                   MEASURE NEW WEIGHT
1090 ENTER 709 : WTA
1100 IF WTA = WTD THEN GOTO 1120 !                            COMPARE WEIGHTS
1110 GOTO 1030 !                                              RESTART HARVESTING LOOP
1120 OUTPUT 709 :"OPN01" !                                   CLOSE HARVESTING VALVE
1130 WAIT 5000
1140 OUTPUT 709 :"DCV02"
1150 ENTER 709 ; WTF
1160 VCOR=WTF-WTD !                                          CALCULATE CORRECTION VOLUME
1170 NCOUNT1=NCOUNT1+1
1180 NCOUNT2=NCOUNT2+1
1190 GOTO 780
1200 OUTPUT 709 :"CLS10" !                                   OPEN DOSING VALVE
1210 OUTPUT 709 :"CLS11" !                                   OPEN SAMPLE VALVE
1220 MTIME=TIME/60 !                                         CALCULATE CYCLE TIME
1230 SETTIME 0.1 !                                           RESET CLOCK
1240 WAIT 300000 !                                           WAIT FIVE MINUTES
1250 OUTPUT 709 ;"OPN10" !                                   CLOSE DOSING VALVE
1260 OUTPUT 709 ;"OPN11" !                                   CLOSE SAMPLE VALVE
1270 OUTPUT 709 ;"DCV02"
1280 ENTER 709 : WTF
1290 CCOUNT=CCOUNT+1 !                                       INCREMENT CYCLE COUNTER
1300 OXY3=MINOXY*2.5
1310 PRINT USING 1320 : "CYCLE ".CCOUNT." COMPLETED IN".MTIME." MIN; D.OXY =".O
XY3." % SAT".WTFV." ML FOAM"
1320 IMAGE 6A.DDD.17A.DDDD.13A.DD.D.6A,DDDD.8A
1330 BYPASS 0
1340 GOTO 780 !                                              CYCLE IS REPEATED
1350 END
```

```
10 REM *** PHASING CONTROL PROGRAME VERSION 12 ***   DEC  01. 1988
20 VCOR1=0
30 LCOUNT=0
40 COUNT1=1
50 NCOUNT=0
60 VCOR2=0
70 NCOUNT2=0
80 SVALVE=0
90 TEST=0
100 MINOXY=2
110 PRINTER IS 701
120 DISP "ENTER TOTAL DOSING VOLUME IN ML"
130 INPUT DVOLT
140 DVOLTV=DVOLT*.0001667 !                             CONVERT VOLUME TO VOLTAGE
150 DISP "ENTER STARVATION PERIOD IN MINUTES"
160 INPUT MSTARVE
170 SSTARVE=MSTARVE*60000+1
180 DISP "ENTER MAXIMUM CONCENTRATION OF NUTRIENT IN MOLES/L"
190 INPUT MAXCON2
200 DISP "ENTER MINIMUM CONCENTRATION OF NUTRIENT IN MOLES/L"
210 INPUT MINCON2
220 DISP "ENTER CYCLE PERIOD IN NO. OF CELL GENERATIONS"
230 INPUT PERIOD2
240 FREQ2=PERIOD2/2
250 DISP "ENTER CURRENT TIME"
260 DISP "USING FORMAT :HOURS. MINUTES. SECONDS SINCE MIDNIGHT"
270 INPUT HRS,MINU,SECS
280 A=HRS*3600+MINU*60+SECS
290 DISP "ENTER DATE USING FORMAT MM.DD"
300 INPUT M,D
310 PRINT USING "16A,X,DD,X,3A,X,DD,X,11A,X,DD,9A,X,DD" : "PHASING STARTED AT",H
RS,"HRS",MINU,"MINS ON DAY",D," OF MONTH",M
320 PRINT USING "21A,X,DD,X,21A,X,D.DDE,X,10A,X,D.DDE" ; "SQUARE WAVE PERIOD IS"
,PERIOD2,"CELL CYCLES: MAX CONC",MAXCON2,": MIN CONC",MINCON2
330 SETTIME 0,1
340 OUTPUT 709 ;"DCV3"
350 ENTER 709 : WITF
360 GOTO 650
370 OUTPUT 709 ;"DCV3"
380 ENTER 709 : WTI
390 IF NCOUNT=PERIOD2 THEN NCOUNT=0
400 IF NCOUNT = FREQ2 THEN GOTO 530
410 WITD=WTI+DVOLTV-VCOR1
420 OUTPUT 709 ;"CLS00" !                           OPEN MEDIUM VALVE 800 (FIRST)
430 OUTPUT 709 ;"DCV3"
440 ENTER 709 : WITA
450 IF WITA = WITD THEN GOTO 470
460 GOTO 370
470 OUTPUT 709 ;"OPN00" !                           CLOSE MEDIUM VALVE 800
480 WAIT 5000 !                                     PAUSE 5 SECONDS
490 OUTPUT 709 ;"DCV3"
500 ENTER 709 : WITA
510 VCOR1=WITA-WITD
520 GOTO 1080
530 WITD=WTI+DVOLTV-VCOR1
540 OUTPUT 709 ;"CLS21" !                           OPEN MEDIUM VALVE 821 (2ND)
550 OUTPUT 709 ;"DCV3"
560 ENTER 709 : WITB
570 IF WITB = WITD THEN GOTO 590
```

```
580 GOTO 550
590 OUTPUT 709 :"OPN01" '                   CLOSE MEDIUM VALVE #1
600 WAIT 5000 '                             PAUSE 5 SECONDS
610 OUTPUT 709 :"DCV2"
620 ENTER 709 : WTTB
630 VLOF1=WTTB-WTTD
640 GOTO 1080
650 ON TIMER# 1,3600000 GOTO 670 '          WAIT 60 MINUTES
660 GOTO 660 '                              IDLE LOOP
670 OFF TIMER# 1
680 MINOXY=2 '                              RESET MINIMUM OXYGEN
690 SCOUNT=0 '                              SET COUNTER
700 OXY1A=0 '                               SET OXYGEN TOTAL
710 SCOUNT=SCOUNT+1 '                       INCREMENT COUNTER
720 OUTPUT 709 :"DCV1" '                    MEASURE OXYGEN
730 ENTER 709 : OXY1
740 OXY1A=OXY1A+OXY1 '                      SUM TOTAL
750 IF SCOUNT=200 THEN GOTO 770 '           CHECK COUNTER
760 GOTO 710
770 OXY1=OXY1A/SCOUNT '                     COMPUTE AVERAGE OXYGEN
780 IF OXY1<MINOXY THEN GOTO 820 '          IF DECREASING, CONTINUE
790 DELTAOXY=OXY1-MINOXY '                  COMPUTE NET INCREASE
800 IF DELTAOXY>.001 THEN GOTO 850 '        IF INCREASE SUFFICIENT, CYCLE
810 GOTO 650
820 MINOXY=OXY1 '                           RESET MINIMUM OXYGEN
830 TEST=TEST+1
840 GOTO 690 '                              CONTINUE TO START OF LOOP
850 MINOXY=OXY1
860 IF TEST<2 THEN GOTO 690
870 ON TIMER# 2,SSTARVE GOTO 890 '          START STARVATION PERIOD
880 GOTO 880 '                              IDLE LOOP
890 OFF TIMER# 2
900 OUTPUT 709 :"DCV2" '                    MEASURE WEIGHT
910 ENTER 709 : WTTA '                      ASSIGN TO WTTA
920 WTTM=WTTA-WTTF
930 WTTD=WTTF+(VOLT0-VLOF2) '               CALCULATE DESIRED WEIGHT
940 WTTM=WTTM*6000
950 OUTPUT 709 :"CLS01" '                   OPEN HARVESTING VALVE
960 OUTPUT 709 :"DCV02" '                   MEASURE NEW WEIGHT
970 ENTER 709 : WTTA
980 IF WTTA < WTTD THEN GOTO 1000 '         COMPARE WEIGHTS
990 GOTO 960 '                              RESTART HARVESTING LOOP
1000 OUTPUT 709 :"OPN01" '                  CLOSE HARVESTING VALVE
1010 WAIT 5000
1020 OUTPUT 709 :"DCV2"
1030 ENTER 709 : WTTF
1040 VLOF2=WTTF-WTTD '                      CALCULATE CORRECTION VOLUME
1050 NCOUNT=NCOUNT+1
1060 NCOUNT2=NCOUNT2+1
1070 GOTO 670
1080 OUTPUT 709 :"CLS10" '                  OPEN DOSING VALVE
1090 DEFINE TIME 60
1100 SETTIME 0,1
1110 IF NCOUNT2=2 THEN GOTO 1190 '          CHECK COUNTER
1120 OUTPUT 709 :"CLS11" '                  OPEN FIRST SAMPLE VALVE
1150 WAIT 300000 '                          WAIT FIVE MINUTES
1160 OUTPUT 709 :"OPN10" '                  CLOSE DOSING VALVE
1170 OUTPUT 709 :"OPN11" '                  CLOSE FIRST SAMPLE VALVE
1180 GOTO 1250
1190 OUTPUT 709 :"CLS12" '                  OPEN SECOND SAMPLE VALVE
```

```
1200 NCOUNT=0 !                                          RESET COUNTER
1230 WAIT 300000
1240 OUTPUT 709 ;"OR11 " !                               CLOSE DOSING VALVE
1250 OUTPUT 709 ;"OR1200" !                              CLOSE SECOND SAMPLING VALVE
1260 OUTPUT 709 ;"DC 03"
1270 ENTER 709 ; WTF
1280 CCOUNT=CCOUNT+1 !                                   INCREMENT CYCLE COUNTER
1290 OXY3=MINOXY*92.5
1300 PRINT USING 1310 ; "CYCLE ",CCOUNT," COMPLETED IN",ITIME," MIN; P.OXY ",OX
Y3," % SAT",WTF," ML FOAM"
1310 IMAGE 6A,DDD,13A,DDDD,13A,DD.D,5A,DDDD,8A
1320 BYPASS=0
1330 GOTO 650 !                                           CYCLE IS REPEATED
1340 END
```

APPENDIX D

CHEMICAL SUPPLIERS

Company	Chemical	Catalogue No.
1) A&C American Chemicals	Ammonium phosphate dibasic	A-252
	Ammonium hydroxide (28%)	A-2345
	Brom cresol green	B-235
	a - napthol	N-078
	Ethanol (85%)	E-146
	Ethylene diamine tetraacetic acid	E-154
	Iodine (0.1N)	I-115
	Glucose	G-152
	Methanol	M-270
	Chloroform	C-270
	Sulphuric acid (96%)	S-499
	Sodium carbonate	S-228
	Ninhydrin	N-162
	Magnesium sulfate ($\cdot 7H_2O$)	M-103
	Sodium phosphate dibasic	S-384
	Potassium phosphate monobasic	P-467
	Sodium thiosulphate	S-445
2) Fisher Scientific Co	Sodium hydroxide (0.5N)	SO-S-270
	Acetic acid (glacial)	A38-214
	Manganese sulphate ($\cdot H_2O$)	M-113
	Ferric sulphate ($\cdot 7H_2O$)	I-146
	Calcium chloride ($\cdot 2H_2O$)	C77-500
	Methyl red	M-219
	Boric acid	A-74
	Soluble starch	S-516
	Sodium sulfite	S-447
3) Anachemia Chemicals Ltd.	Sodium dodecyl sulphate	AC-3950
	Cupric sulphate	AC-2675
4) Sigma Chemical Co	Dodecyltrimethyl ammonium bromide	D-8638