STUDIES ON THE RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING WASTEWATER PURIFICATION BY ACTIVATED SLUDGE

By

G. JEYASINGH THABARAJ

Bachelor of Science
University of Madras
Madras, India
1956

Bachelor of Science in Technology
University of Madras
Madras, India
1959

Master of Science
University of Madras
Madras, India
1962

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF Philosophy
August, 1969

STUDIES ON THE RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING WASTEWATER PURIFICATION BY ACTIVATED SLUDGE

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGEMENTS

It is a pleasure for the author to acknowledge his gratitude to the following people, without whose help this dissertation would not have materialized:

Recognition is given first to his major adviser, Dr. Anthony F. Gaudy, Jr., for his constant inspiration, encouragement, and guidance throughout the course of this investigation.

Dr. Elizabeth T. Gaudy gave generously of her time in reading the manuscript and offering valuable suggestions. Her help in microscopy is gratefully acknowledged.

Dr. Don F. Kincannon kindly consented to serve on the author's advisory committee, and offered helpful comments on the manuscript.

Appreciation is due to Professor Q. B. Graves and Dr. K. E. Ebner for serving on the advisory committee.

The author wishes to acknowledge the cooperation and help rendered by all of his colleagues in the Bioenvironmental Engineering Laboratories, especially by Mr. J. A. Heidman.

Mrs. Grayce Wynd deserves special thanks for her painstaking effort in carefully and accurately typing the thesis, and for her willingness to offer help whenever needed.

Thanks are due to Mr. Eldon Hardy for his help during the final preparation of the figures.

Lastly, but by no means least, the author is indebted to his wife

and parents for their patient understanding, encouragement, and sacrifice.

Financial support for this study was provided by research grants WP00075 and WP00325, both from the Federal Water Pollution Control Administration, U. S. Department of the Interior.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

A. Nature and Importance of the Problem

The average annual streamflow discharging into the oceans from the continental United States is approximately 1100 billion gallons per day (BGD). Approximately 300 BGD are now withdrawn for municipal and industrial uses, power generation, and irrigation. However, it has been predicted that by the year 2000 AD, polluted return flows will reach about two-thirds of the entire streamflow (1). Urbanization and industrialization have added to the pollution problem by concentrating the wastes and by increasing the quantity of waste per individual. The U. S. Public Health Service has estimated that by the year 2000, 95 per cent of the total population of some 280 million people will reside in urban areas, and that the wastes discharged through municipal sewer systems will average about 132 gallons per day per person (1). Industrial expansion since World War II has aggravated the pollution problem by increasing the types and amounts of pollutants. It is estimated that the contribution from industrial wastes to the organic wasteload handled by municipal waste treatment systems will have a population equivalent equal to one-third of the total organic load in the United States by the year 2000 (1).

As the need for greater quantity and the deterioration of the

quality of the water become more critical, the growing demands for fresh water can be met only by re-use of the water resource. The burgeoning population and the increase in per capita water consumption are making re-use of water a necessity. It has been reported that in 1963, of the 180 million people in the United States, 70 million were using water derived from domestic sewage and industrial waste effluents (2). All waste waters returned to the streams must be given adequate treatment to remove any pollutants which would degrade the water and make it unfit for another required use downstream.

The purification capabilities of conventional treatment units (primary and secondary) are limited, and the "secondary" effluents discharged into the streams contain soluble and suspended organic matter and inorganic nutrients such as nitrogen and phosphorus, which can promote overproduction of biota in the receiving streams. The functions envisioned for "advanced waste treatment" (now being researched extensively) include removal of residual organic and inorganic materials from secondary effluents to such an extent that they may be immediately re-used (3).

Often the cost of treatment of waterborne wastes can be reduced if wastes are treated in large volumes. Considerable economy can be effected if industrial wastes are combined with municipal wastes, provided combination of the wastes does not create major operational problems during treatment. Municipal wastes can supply essential microbial nutrients, such as nitrogen and phosphorus, and thus can provide a suitable nutritional balance if wastes deficient in these nutrients are added (4).

Many industrial wastes (e.g., canesugar refinery, textile, pulp and

paper mill wastes) are deficient in nitrogen, and it is necessary to add supplemental nitrogen before biological treatment if combined treatment with domestic wastes is not feasible. For wastes totally deficient in nitrogen, the cost for this supplementation may be comparable to that of air compressor power costs when the activated sludge process is used (5). The need for a high degree of waste treatment has increased the popularity of the activated sludge process, which is one of the few waste treatment processes which can produce a high quality effluent at a reasonable cost (6). Also, this process offers the greatest opportunity for a more enlightened engineering approach. Extensive research in the Bioenvironmental Engineering Laboratories of Oklahoma State University has led to a modification of the activated sludge process for the treatment of nitrogen-deficient wastes (7). Successful separation of the "growth" (sludge accumulation) and "replication" phases of substrate utilization was demonstrated. Further studies have shown that continuous oxidative assimilation of the carbon source and the regeneration of the sludge in the presence of a nitrogen source can be accomplished in separate unit operations (8)(9). The waste sludge produced in this process is rich in non-nitrogenous materials, and little is known concerning its aerobic digestion characteristics.

It has been reported that the excess sludge produced in conventional activated sludge processes is not readily susceptible to anaerobic digestion, since much of the sludge mass is inert material and living microbes which are not destroyed readily by anaerobic digestion (6). Therefore more emphasis is currently placed on aerobic digestion of excess sludge. Information is available on the autodigestion

characteristics of conventional activated sludge due largely to research accomplished on the extended aeration process. The consensus of a majority of researchers in the field is that even sludges developed under balanced growth conditions cannot undergo total "endogenous" oxidation (10)(11)(12)(13)(14). Only scant information is available on the endogenous oxidation of sludges developed under nitrogen deficiency. Accumulation of large amounts of biologically-resistant extracellular polysaccharide during metabolism of nitrogen-deficient activated sludge systems operating without sludge wasting has been reported (13). However, the factors contributing to improved autoxidation of such sludges are not well understood. Major concerns in the field of biological treatment are delineation of the metabolic character of endogenous metabolism and the extent of endogenous oxidation of sludges developed in the presence or absence of exogenous nitrogen. Since the nature of the excess sludge would be drastically different according to the availability of nitrogen (15), it seems necessary to assess the changes in biochemical composition of the sludge during solids accumulation and endogenous phases in order to understand the mechanistic aspects of autoxidation.

One of the operational problems in activated sludge treatment (especially when industrial and municipal wastes are combined) is the occasional overload or shock loadings imposed on the plant due to large and unpredictable variations in strength and volume of wastes. Shock loads are defined as any rapidly occurring or immediate changes in the chemical or physical environment imposed on a biological waste treatment system (16). The term "quantitative" shock load has been used to denote a change in the amount of inflowing organic substrate, a rapid

increase or decrease in organic loading due to an influx of a higher or lower concentration of the substrate to which the organisms were acclimated. Several aspects of quantitative shock loads have been the subject of recent studies in the Bioenvironmental Engineering Laboratories of Oklahoma State University (17)(18). Conditions are known which could cause interim disruption of the activated sludge process and lead to high effluent COD due to the release of metabolic intermediates and/or endproducts. One aspect of the process in which further research was warranted is the possible effect of low dissolved oxygen concentration on the metabolic response of the organisms to the shock loading.

In view of the recent interest in advanced waste treatment and water renovation, it is necessary to understand the nature of pollutants present in the secondary effluents. Many of these pollutants may be derived from the metabolism of the various substrates by the heterogeneous microbial populations during treatment. Such metabolic products may at times accumulate and be subsequently metabolized, or they may persist in the effluent, depending upon the conditions prevailing during treatment. Of significance in this regard are the lower aliphatic acids which may appear as metabolic products regardless of whether the original substrate was carbohydrate, protein, or lipid; such products have been identified in secondary effluents and river waters (19).

In various studies conducted at the Bioenvironmental Engineering Laboratories of Oklahoma State University it has been shown that volatile fatty acids are produced by heterogeneous microbial populations during the metabolism of the original exogenous substrates. Because of the importance of this phenomenon in determining both the overall mechanism and kinetics of waste water purification, a detailed study aimed

at determining the generality of occurrence of intermediary metabolites or endproducts and the conditions which foster or prevent the release of such products seemed warranted.

B. Scope of the Present Investigation

The present study is concerned with the influence of various environmental factors on the nature and extent of elaboration and accumulation of metabolic intermediates and/or endproducts during the metabolism of heterogeneous microbial populations present in activated sludge. The effect of the following factors, which have fundamental ramifications to the design and operation of conventional as well as modified activated sludge systems, were examined in this investigation:

1. dissolved oxygen tension
2. quantitative shock loads
3. nitrogen deficiency
4. initial COD to biological solids ratio
5. nature of carbon and energy sources
6. prolonged or extended aeration.

The first phase of this study was designed to evaluate the individual and combined effects of the first two factors on the metabolic response of a completely mixed continuous flow activated sludge process. Glucose was used as the carbon and energy source for these experiments.

The second phase involved the delineation of the effects of factors 3 through 6 on the nature and amounts of accumulation of biological solids and metabolic products during waste purification in batch-fed systems. Short-term batch experiments were conducted to assess the individual and combined effects of factors 3 and 4 on systems with different carbon and energy sources. Extended aeration studies on once-fed

batch systems were performed in order to evaluate the individual and combined effects of factors 3 through 6.

Attempts were also made to define (by qualitative and quantitative analyses) the specific metabolic intermediates and/or endproducts which were produced under each operational condition. It was the aim of the study to seek possible correlations between the amount and nature of metabolic products and the environmental or operational conditions, in order to provide further insight into means of control of the activated sludge process. It was felt that even if the study did not lead to drastic changes in the design or operation of activated sludge systems, it would contribute to a better understanding of the kinetics and mechanisms operative in activated sludge systems under both steady and changing environmental conditions.

CHAPTER II

THEORETICAL ASPECTS OF MICROBIAL GROWTH AND METABOLISM

A. Growth Cycle of Bacteria

When a small number of viable bacterial cells is placed in a medium containing excessive food supply in a suitable environment, conditions are established in which unrestricted growth takes place. The new cell material synthesized grows (replicates) at an essentially constant doubling time and the population attains the "exponential" phase. However, as the number of organisms increases, competition between individual organisms for the available food supply reduces the opportunity for further growth, the growth rate decreases and eventually stops, at which time the "stationary phase" is attained. The stationary phase is followed by a phase of decline in population density. First, the utilization of internal food reserves occurs, and finally the structural components of the organisms may serve as sources of energy for the continued respiratory activity of the organisms (20). Very little is known of the kinetics of bacterial death after exhaustion of the available extra- and intracellular food supply.

B. Nutritional Requirements of Bacteria

The nutritional requirements of heterotrophic bacteria vary widely. Apart from an organic carbon and energy source, they require nitrogen, phosphorus, and sulfur in appreciable amounts. The

nutritional aspects of activated sludge treatment have been reviewed by Sawyer (21), who recommends a 5-day BOD:N ratio of 17 to 1, and BOD:P ratio of 90 to 1 when it is desirable to produce a biological growth or activated sludge with maximum nitrogen and phosphorus content. The requirements for sulfur and other trace elements are met by the carriage water.

C. Bacterial Metabolism

1. General Comments

Bacteria have a far greater metabolic flexibility than do cells of higher organisms, and under different nutritional conditions a given bacterium may exhibit a diversity of metabolic pathways. In the aerobic metabolism of a carbon compound two inseparable metabolic activities take place:

i) The carbon source is oxidized to furnish the energy (ATP) needed for life and growth, and

ii) A portion of the carbon source is used as "carbon skeletons" for the synthesis of new cellular components, e.g., proteins, carbohydrates, nucleic acids, and lipids. Figure 1 shows the biochemical transformations of the substrate during growth and endogenous phases in aerobic heterotrophic organisms. The relative proportions of these transformations depend upon the type of microorganisms, and the physical and chemical conditions of the immediate environment which influence microbial growth and metabolism.

In the treatment of wastes by activated sludge, no direct control can be exercised regarding the selection of the types of organisms. The types of species which will predominate depend upon the nature of substrates and the growth conditions. However, judicial selection of the

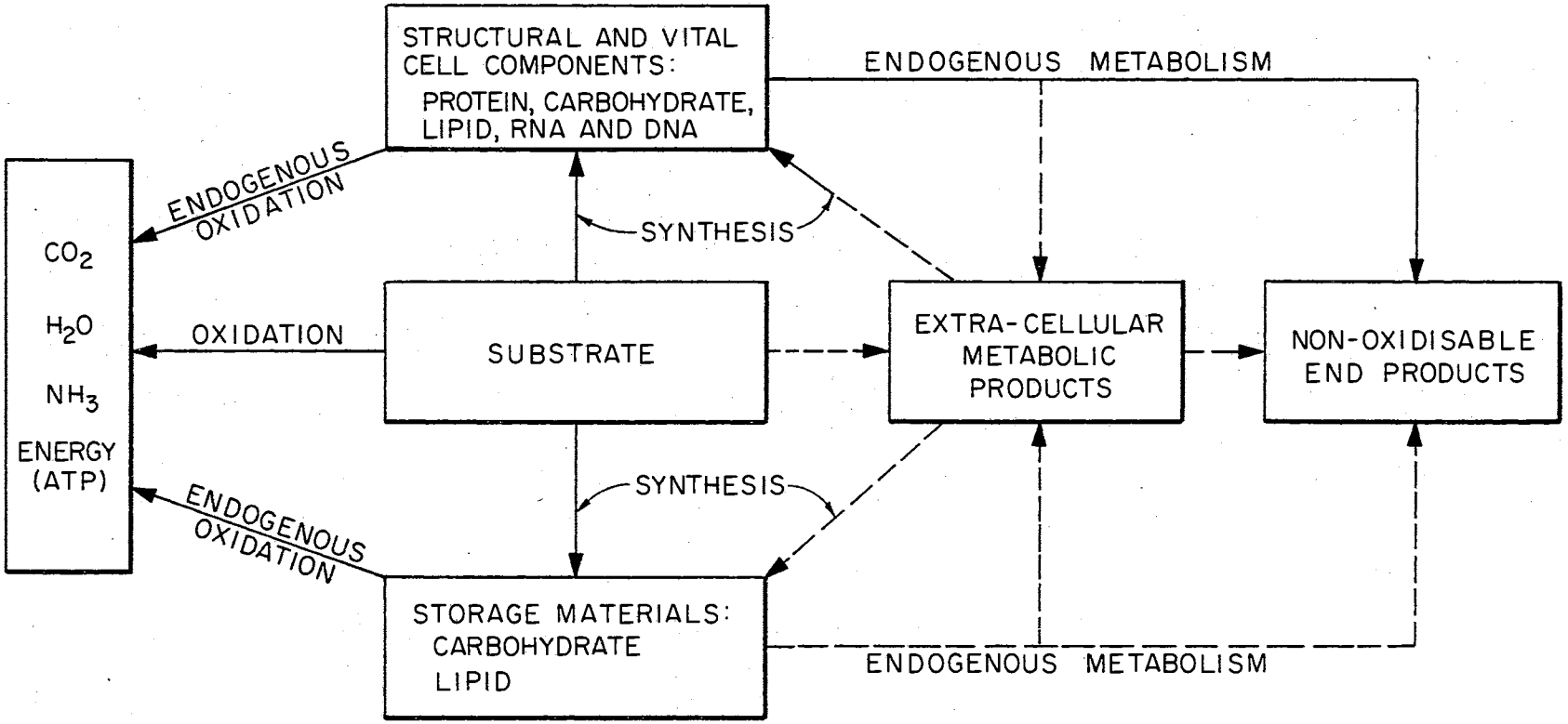


Figure 1 - Biochemical transformations of substrate during growth of aerobic heterotrophic organisms.

growth conditions can ensure maximum efficiency with respect to substrate removal and solids production.

2. Metabolism of Nitrogen-deficient Substrates by Bacteria

a. Uncoupling of Energy-producing and Energy-consuming Reactions

In order that a constant yield of cells is formed per mole of ATP produced, an effective coupling mechanism between the energy-yielding oxidations and the energy-consuming biosynthetic reactions of the cells is necessary. In some systems such a balanced coupling may be achieved if the growth rate is limited by the speed of energy production from the substrate. If, however, the growth rate is limited by the rate of the biosynthetic processes, energy would be produced in excess and wasted (uncoupling) unless the cell is able to control the production of energy by the rate of its utilization. This type of regulation has not been demonstrated in growing bacteria (22). The fact that several organisms can assimilate substrates in the absence of exogenous nitrogen shows the ability of the organisms to metabolize the substrates in the absence of a balanced synthesis of all cell components and demonstrates the lack of adequate coupling between the catabolic and anabolic functions. Under these conditions the energy normally channelled into synthesis of nitrogenous polymeric compounds may be utilized in the following ways (23):

i) accumulation of polymerized products either in a storage form or as unusable waste

ii) activation of alternative pathways bypassing energy-yielding reactions or consuming all of the available energy for priming (initiation of a biochemical reaction), and

iii) dissipation as heat by the "adenosine triphosphatase" systems.

b. Metabolic Shift due to Nitrogen Deficiency

The primary metabolic pathways of microorganisms are intrinsically adjusted to accomplish growth by the replication of the cell. Deficiency in an essential nutrient such as nitrogen causes the dislocation of this delicate adjustment. As a result of this dislocation, certain key metabolic intermediates and cofactors such as ATP (24), UDPG (25), and glycolytic intermediates (26) not being utilized for the synthesis of nitrogenous macromolecules, attain high levels. Various enzymes responsible for the synthesis of storage compounds, e.g., carbohydrates and lipids, become active due to induction, derepression, activation, or increased availability of the required substrates which had been hitherto limited by the effective competition of primary metabolic pathways (25) (26). In the absence of cell division, the cells may become larger due to the accumulation of these storage materials, and the surface to volume ratio of the cell decreases. Access of nutrients from outside may become increasingly difficult.

These secondary processes of enzyme activity, the pace of which is accelerated in nitrogen-deficient systems, may themselves be liable to control mechanisms such as endproduct inhibition. When the physical limit to which a given cell can accommodate a storage product is reached, the process may stop.

c. Control of Carbohydrate Synthesis During Nitrogen-limited Growth

One of the important factors that control the synthesis of glycogen in bacteria is the concentration of nucleotides in the cell. Nucleotides are involved in the critical steps in gluconeogenesis. Under conditions of nitrogen deficiency in the presence of excess carbon source,

the intracellular level of ATP increases in bacteria (24) due to the fact that the rate of its utilization for synthesis of new macromolecules is lower than its rate of production by the uninterrupted catabolism of carbon source. Glycogen accumulation under such conditions has been shown to be a result of excess ATP production and accumulation of glycolytic intermediates (26).

Different mechanisms of glycogen synthesis in bacteria have been proposed. Madsen (25) found that UDPG was the glucosyl donor for glycogen synthetase in Agrobacterium tumefaciens. UDPG also inhibited the glycogen-degrading enzyme, phosphorylase, in this organism. The changes in the concentration of glycogen during nitrogen-limited growth of the organism were accmpanied by parallel changes in UDPG concentration. The concentration of the latter was raised due to increased availability of ATP (and hence UTP) and hexose phosphates resulting from the continued metabolism of the carbon source even after depletion of nitrogen from the medium. Madsen suggested that the uridine base needed under these conditions may be derived from the breakdown of nitrogenous reserve materials like RNA and protein.

Regulation of synthesis of glycogen in Escherichia coli B has been found by Preiss, et al. (26) to be controlled by the activity of ADP-glucose pyrosphosphorylase which is activated by several glycolytic intermediates, fructose -1,6-diphosphate being the most effective. They observed that the glucosyl donor for glycogen synthetase in Escherichia coli B was ADP-glucose; its synthesis from ATP and glucose-1-P is mediated by ADP-glucose pyrophosphorylase. This enzyme is activated by ATP as well as by glycolytic intermediates which would accumulate when balanced growth in the presence of excess substrate is

limited by nitrogen. On the other hand, utilization of ATP at a rapid rate would lead to increases in the concentrations of ADP, AMP, and Pi, which would inhibit the pyrophosphorylase and therefore synthesis of ADP-glucose and glycogen. Other glycolytic intermediates such as phosphoenolpyruvate, glyceraldehyde-3-P, fructose -6-P, ribose-5-P and pyruvate also activate the enzyme. These findings are in accord with the effect of adenine nucleotides (ATP, AMP, ADP, or 5'-AMP), which regulate the activity of phosphofructokinase and NAD-specific isocitrate dehydrogenase which are involved in the utilization of carbohydrates to form ATP.

3. Endogenous Metabolism

The ultimate aim of any biological waste treatment process is to convert the organic components into stable endproducts like CO_2, H_2O, NH_3 and sludge. Sludge disposal is a major economic consideration in the design of waste treatment facilities. If no effective and economical utilization of the excess sludge is feasible, means should be found to completely oxidize the sludge or to reduce its volume by biological action. One way of accomplishing this is to aerate the sludge in the absence of nutrients for an extended period of time, during which the organisms oxidize their own cell material (endogenous metabolism) and die (lyse).

Endogenous metabolism is defined as the "sum of all activities performed by the organisms in the absence of utilizable extracellular materials serving as sources of energy and building stones for assimilation and growth" (27). It has been stated that the role of endogenous metabolism is to provide the energy needed for the maintenance of the cell and to preserve its viability until eventual death (28).

Experimental evidence for the requirement of energy of maintenance has been provided by McGrew and Mallette (29), and Marr, et al. (30).

It is not known, however, whether endogenous metabolism can be sustained for an indefinite time without impairing the essential functions of the cell. It is also not known to what extent the energy-yielding reactions of endogenous metabolism contribute to the survival of cells. Even though endogenous metabolism results in ATP formation, no correlation between bacterial ATP content and survival has been observed (31).

Since the extended aeration process makes use of the endogenous metabolism of sludge organisms in order to reduce the weight of the sludge and therefore to obviate the need for separate sludge treatment, the factors influencing endogenous metabolism, death and lysis are of much significance in the successful aerobic oxidation of the sludge.

4. Death and Lysis

Microbes do not suffer "natural" death as observed in higher organisms. Death in microorganisms is caused by exposure to an unfavorable physical or chemical environment such as prolonged exposure to a nutrient-deficient environment where balanced growth and replication is prevented by withholding one or more essential nutrients in an otherwise complete medium. "Death" under these conditions is due to the destruction or inactivation of enzymes and co-enzymes which cause the cell, irreversibly, to lose its growth potential. The term "lysis" implies dissolution of the cell due either to natural causes (autolysis) or with the aid of an exogenous agent (32). Neither the causative factors nor the mechanism of cell lysis is clearly understood. Autolysis is believed to be initiated whenever the metabolism of the cell is disturbed drastically, e.g., exhaustion of carbon source (33). After the loss of

vital functions of metabolism, the autolytic enzymes are believed to act upon the cell and cause lysis. These enzymes can also be liberated and can act on other cells in the environment (32)(34). It is not known what triggers the action of autolytic enzymes. Apart from autolytic enzymes, various other products of microbial origin have also been reported to cause lysis or dissolution of living bacteria, dead cells, and cell walls (32).

5. "Cryptic Growth" Among Starving Bacteria

It has been observed that when a microbial population is starved, the leakage and lysis products of the dead cells support the growth of some of the survivors. This phenomenon is known as "cryptic growth" (35). Postgate and Hunter (36) observed that the death of fifty members of a starved population of Aerobacter aerogenes, derived from a chemostat, allowed the doubling of one survivor. According to Postgate (37), "cryptic growth" may be an important factor in studies involving addition of a carbon source to a starved population (i.e., oxidative assimilation studies) because under such situations death of one cell may support the growth of another. Thus, in such "nonproliferating" system, turnover of protein can occur.

6. "Population Effect" Among Dying Bacteria

Death under starvation conditions has been found to be more rapid in sparse bacterial populations than in denser ones (38)(39). Postgate and Hunter (39), and Harrison (38) surmised that dying cells secreted materials which, when they exceeded a threshold concentration, enabled their surviving neighbors to maintain viability longer than they otherwise could. Harrison states, however, that this population effect can be inverted at high bacterial densities due to anoxia (insufficient

supply of oxygen).

7. Oxygen Tension and Microbial Metabolism

Molecular oxygen serves as the ultimate acceptor of hydrogen ions and electrons in the terminal respiration of organic substrates by aerobic organisms. There is evidence that low oxygen tensions can affect the metabolic pathways used (40)(41)(42), and the cytochrome content (43)(44) of microorganisms. Oxygen tension may also mediate the effects of chemical and environmental stress on cells (45).

It is known that the respiration rate (mg O_2/hr/gm dry wt) of cells is independent of the dissolved oxygen tension of the medium so long as the latter remains above the "critical O_2 tension," the value below which the respiration rate begins to decrease. The decrease in respiration rate at low oxygen tensions has been attributed to unsaturation of enzyme surfaces (46) and/or restricted oxygen diffusion through cell substance (47)(48). At high oxygen concentrations, where oxygen uptake rate is independent of the oxygen concentration, the supply of intermediary metabolites (e.g., ADP and reduced NAD)(48), and/or the resistance to transfer of oxygen across the barrier provided by the cell-liquid interface (49)(50) may limit the respiration rate. Apart from the effect of low O_2 tensions, the respiration rate of microorganisms is a function of the nature of the populations, their growth rate (51), and the physical and chemical factors of the environment (52).

Of practical significance to the activated sludge process is the effect of oxygen tension on purification efficiency. Since decreased respiratory activity of the organisms would lead to only partial oxidation of the wastes, it is desirable to know the minimum DO level that would satisfy the metabolic requirements of the organisms and ensure the

desired degree of substrate removal.

8. Release of Metabolic Intermediates and Endproducts

Metabolic intermediates or endproducts are compounds formed by metabolic conversions of the primary substrates and are disposed of by the organisms by excretion into their immediate environment either transiently or permanently. The metabolism of carbon and energy sources by bacteria involves a series of reactions catalyzed by enzymes whose activity is subject to metabolic control mechanisms. The various intermediates and endproducts secreted by the organisms arise from the normal or altered activities of the enzymes of the metabolic pathways. The factors responsible for the accumulation of such compounds are summarized below.

a. Genetic Factor (or Type of Organism)

i) Altered Feedback Control

Either a decreased level of feedback inhibitor due to the existence of a metabolic block after a preceding intermediate or decreased response to the endproduct may cause an organism to secrete the intermediate or endproduct of a metabolic pathway (53). Such organisms which excrete the endproduct are used in Japan for the production of glutamic or other amino acids.

ii) Repression and Induction of Enzymes During the Growth Cycle

Transient repression of the synthesis of enzymes for the oxidation of acetate and pyruvate during vegetative growth and their induction during transition to sporulation have been observed in Bacillus subtilis by Nakata and Halvorson (54), Hansen, et al. (55), and Szulmajster and Hansen (56). During vegetative growth, large amounts of acetic and

pyruvic acids accumulated in the medium. Similar regulatory mechanisms may be operative in another species (tentatively identified as Escherichia intermedia) which accumulates volatile acids during growth on glucose in a minimal medium and oxidizes them after exhaustion of glucose from the medium (57).

b. Environmental Factors

i) Type of Carbon and Energy Source

A significant feature of microbial growth on glucose is the rapidity with which it is metabolized by the enzyme system already present in the cells (58)(59)(60). This is accompanied by the formation of intermediates at a much faster rate than they can be removed by anabolic reactions, and consequently intermediates are excreted into the medium (59).

Repression of the synthesis of several TCA cycle enzymes and enzymes of the glyoxylate bypass during aerobic growth of many microorganisms on glucose in minimal or enriched media has been reported by many workers (61)(62)(63)(64)(65)(66). Under such conditions, glycolytic reactions function rapidly in relation to terminal respiration, and acetic (and pyruvic) acids accumulate in the medium (61)(67). Partial alleviation of glucose repression can occur when bacteria are grown in a minimal medium in which the TCA cycle must be used for synthetic purposes (62). Repression may also be reversed by sufficient concentration of accumulated metabolites (63).

A high energy compound produced during the rapid glycolytic utilization of glucose is implied as the repressor for the enzymes required for terminal oxidation (68). Changes in the concentration of adenosine phosphates and nicotinamide nucleotides during aerobic growth of

Saccharomyces cerevisiae on glucose and galactose (66) suggest ADP as the probable deactivator of the repressor molecule which governs the production of respiratory enzymes.

ii) Nutritional Deficiency

Occurrence of a partial metabolic block at a key intermediate stage in the metabolic pathway during nitrogen-limited growth of Pseudomonas aeruginosa is suggested by Duncan and Campbell (69). Since the hexose monophosphate pathway may be less active during metabolism of glucose in the absence of exogenous nitrogen (70), more substrate flows in the glycolytic pathway, and this may cause increased repression of TCA cycle enzymes. The portion of the substrate channelled to the glycolytic pathway can be reduced if the cells activate their glycogen synthesis systems and thus control the flow of substrate through the glycolytic pathway. If the cells are unable to conserve the carbon source in the form of a stored product, intermediates would be excreted into the medium.

iii) Availability of Oxygen

Adaptive development of cytochromes (43)(71) and induction and repression of TCA cycle enzymes (72) in response to access of oxygen have been observed in facultative anaerobic bacteria. If cytochrome content is lowered, shifts to lowered efficiency of assimilation (fewer ATP's from flavine oxidation) and to increased use of electron acceptors other than O_2 might occur (73).

9. Continuous Culture of Microorganisms

a. "Steady State" Growth

During the exponential growth phase of a bacterial culture, the

cells are replicating at a constant doubling time, representing an approximate and limited steady state in regard to the relation between the bacteria and their external environment. The rate of growth in the exponential phase is limited either by the biochemical potential of the cell or by the concentration of an essential nutrient or oxidizable substrate. Bacterial growth in the exponential phase may be maintained by rapid successive transfers from this phase to fresh medium or by continuous addition of sterile medium. In a completely mixed continuous culture system, the sterile nutrient medium is supplied at a constant rate and the volume of the system is kept constant by withdrawing the suspension at the same rate.

The increase in cell mass during exponential growth of a bacterial culture can be expressed by the equation

$$\frac{dx}{dt} = \mu x$$

where x is the dry weight of organisms per unit volume at time t, and μ is the exponential or specific growth rate. The relationship between specific growth rate and substrate concentration was found to be a "saturation function," the value of μ asymptotically approaching a maximum value (μ_m) with increasing substrate concentration (74).

If f is the constant flow rate of nutrient medium fed into a continuous culture system, the dilution rate D may be expressed as f/v, where v is the volume of the culture vessel. The rate at which the cells are leaving the completely mixed system would then be

$$-\frac{dx}{dt} = Dx$$

The net increase in bacterial mass in the system would be

$$\frac{dx}{dt} = (\mu - D)x$$

"Steady state" can be obtained if

$$\mu = D$$

i.e., if the flow rate is equal to the specific growth rate. Since the growth rate in a bacterial culture can be limited by reducing the concentration of one of the essential nutrients in the medium, it is possible to obtain "steady state" over a wide range of growth rates. The concentration of the limiting nutrient is instantaneously reduced when the medium is mixed with the culture and reaches a level that limits the growth rate. The growth rate, then, is determined by the flow rate of the medium, and the bacterial mass is determined by the limiting nutrient (75).

b. Use of a Completely-mixed Continuous Flow System for the Study of the Effect of Oxygen Tension

Continuous culture provides a time-independent and stable environment for the organisms. They can be maintained in a relatively constant physiological state indefinitely, so long as the dilution rate is not altered. The rate of synthesis of every component of the cell is then equal to the dilution rate which can be regulated by the experimenter to any values that do not exceed the maximum value of μ, i.e., the maximum synthetic capacity of the cells (75).

It is difficult to maintain a constant dissolved oxygen concentration in a batch culture, since this depends upon the oxygen demand of the system which increases with cell concentration. But the "steady state" cell concentration in a continuous flow system is fairly constant, and so is the oxygen demand.

c. Dilute-in of Additional COD in a Steady State System due to a Quantitative Shock Load

In the steady state condition prior to a shock loading, the rate of substrate removal due to growth of the microorganism can be expressed as follows:

$$\frac{dS}{dt} = DS_{1_o} - DS_o$$

where S_{1_o} and S_o are the concentrations of the substrate in the feed and in the reactor, respectively, prior to the shock. If the cells do not respond to the shock, i.e., do not remove the additional incoming substrate but continue to remove the carbon source at the same rate as before the shock, the rate of change of substrate concentration due to increase in substrate will then be given as follows (76):

$$\frac{dS}{dt} = DS_1 - DS - D(S_{1_o} - S_o)$$

where S_1 is the concentration of the substrate in the shock feed, and S is the concentration of the substrate in the reactor at time t after initiating the shock.

Integrating the above equation with S_o as the value of S at zero time and solving for S, the following equation for the dilute-in of additional substrate is obtained:

$$S = (S_1 + S_o - S_{1_o}) + (S_{1_o} - S_1)e^{-Dt}$$

The "dilute-in" curve calculated using the equation above can be compared to the observed substrate concentration during the transient phase following the increase in feed, and it is thus possible to determine whether the rate of substrate removal has changed (increased) in response to the shock.

10. Microbial Interactions

Microorganisms differ widely in their specific nutritional requirements, and their ability to grow in a given environment is a function of their genetic makeup as well as the physico-chemical properties of the environment. In natural environments containing diversified sources of food, several species of organisms co-exist; the relative predominance of species is determined by the interactions of the organisms and the prevailing environmental conditions (77).

The factors affecting microbial growth in pure cultures are also operative among mixed populations in natural environments, e.g., sewage and soil. However, an additional factor that has an important bearing in the study of mixed populations is the interaction between different species of organisms. The decomposition of even a simple substrate such as glucose may involve sequential development of specialized populations capable of utilizing the metabolic intermediates or incompletely oxidized products generated by preceding populations (78). Findings on several types of interactions among mixed microbial populations in continuous culture have been reviewed by Bungay and Bungay (79).

Although growth rate is considered to be one factor in the selection of predominant organisms in continuous culture (80)(81), microbial interactions such as inhibition (82), wall growth (82), commensalism (83), mutualism, and predation contribute toward maintaining the heterogeneity of the populations.

11. Summary

Studies on the metabolism of bacteria in a medium lacking a nitrogen source show that cell division is not an obligatory requirement for cell growth. In the absence of effective coupling between energy

production and synthesis of cell components, catabolism of substrate can occur with the resultant accumulation of metabolic intermediates and/or endproducts which can be diverted to the synthesis of non-nitrogenous cellular materials or disposed of into the external medium either transiently or permanently. The nature and extent of elaboration of intermediates and endproducts depend upon the type of organism and the nature of the environmental stress imposed upon the organisms. Complex microbial interactions in a mixed culture may also influence the accumulation and utilization of metabolic intermediates.

CHAPTER III

LITERATURE REVIEW

A. Effect of Oxygen Tension on Microbial Activity

The effect of dissolved oxygen tension on microbial activity has been a subject of much study in the microbiological field. Most of the earlier work on oxygen tension was concerned with the effect of oxygen tension on the respiration rate of microorganisms. Very few studies have been made on the effect of dissolved oxygen on the metabolic fate of substrates. A few selected papers which are of significance in the present investigation will be reviewed in the following pages. Earlier works on the effect of oxygen tension on the activity of pure and mixed cultures have been reviewed by Okun and Lynn (84), Smith (85), Gaden (52), and Gaudy and Turner (86). It is unfortunate that the earlier studies on the effect of oxygen tension on microbes were severely handicapped by the lack of a convenient and reliable means for the measurement of dissolved oxygen concentration. The recent development of membrane electrodes has made possible *in situ* measurement of DO in biological systems.

1. Pure Cultures

a. Critical Oxygen Tension

The "critical oxygen tension," the value below which the respiration rate begins to decrease, varies with the type of organism. The

results of Baumberger (87), Carter and Bull (40), Phillips and Johnson (88), and Harrison and Pirt (89) indicate that the critical oxygen tension in microorganisms is in the range of 0 to 15 mm Hg (about 0 to 0.7 mg/l at 30°C). Harrison and Pirt have, however, shown that the response of Klebsiella aerogenes to decreased oxygen tension involved a stimulation of respiration rather than a decrease. They suggest that a low DO acted as an uncoupler of oxidative phosphorylation.

b. Cell Yield

Smith and Johnson (90) found that the percent yield of Serratia marcescens based on substrate utilized in a synthetic medium containing glucose and citrate varied directly with aeration efficiency. The cell concentration varied from 9 mg/ml at an effective aeration rate of 0.5 mM O_2/l/min to 23 mg/ml at an aeration rate of 9 mM O_2/l/min. They concluded that the factor limiting final cell concentration in their experiments was the oxygen that was available to the organisms. No dissolved oxygen data are available in this study. However, in a later study, Phillips and Johnson (88) demonstrated that no correlation existed between oxygen supply rate and cell yield if the dissolved oxygen level in the medium was maintained above 0.01 to 0.02 atmospheres (0.4 to 0.7 mg/l at 30°C), depending upon the organism used.

The influence of dissolved oxygen tension on the dry weight yield of Klebsiella aerogenes in a steady state continuous culture was studied by Harrison and Pirt (89). With glucose as the growth-limiting substrate, they found that in the "excess oxygen state" (DO tensions above 10 to 15 mm Hg) the cell yield and CO_2 production decreased with decreased oxygen supply.

c. Substrate Metabolism

Correlation between the available oxygen and the types of end-products was observed by Pirt in steady state cultures of Aerobacter cloacae (42). Under anaerobic conditions he observed minimum production of cells and CO_2 while most of the glucose was converted to ethanol, formic acid, 2,3-butanediol, and acetoin. A small supply of oxygen suppressed the formation of ethanol and formic acid, but still permitted production of butanediol and acetoin, and increased the proportion of glucose converted to acetic acid, cells, and CO_2. A larger oxygen supply led to suppression of the formation of butanediol and acetoin, and further increase in cell yield. Complete conversion of glucose into CO_2 and cells and suppression of acetic acid formation was found to occur when excess oxygen was available, provided the growth rate of the organism was not "too near its maximum." When the growth rate was close to maximum, a part of glucose was converted into acetic acid even with an excess of available oxygen. Thus Pirt found differences in the metabolism of the organism under conditions which he characterized as fully aerobic, partially aerobic, and fully anaerobic. He obtained these different conditions by progressively decreasing the oxygen absorption coefficients of the system. He did not, however, measure the dissolved oxygen tensions in these states.

Ecker and Lockhart (41) studied the metabolic events occurring in cultures of Escherichia coli K-12, limited by the availability of oxygen in a defined medium containing glucose and $(NH_4)_2SO_4$. Oxygen limitation was caused by providing an initially low aeration rate. When the oxygen availability decreased to about 6 to 8 x 10^{-2} m moles/cell/hr, cell growth and ammonia nitrogen utilization terminated abruptly.

However, glucose utilization continued at a rapid rate after cessation of growth with a parallel drop in the pH of the medium. It is interesting to note that growth of the population stopped while glucose and nitrogen were available in excess and oxygen was still present. These workers surmised that an adaptation to anaerobic metabolism in this system appeared as a constant shift in emphasis from oxidative to fermentative metabolism (as suggested by drop in pH) occurring gradually as increasing cell population gradually reduced the oxygen available to each cell. In additional experiments they noted a specific level of oxygen availability which, although insufficient for fully aerobic growth, seemed to inhibit anaerobic processes. They suggest that this might be a quantitative manifestation of the Pasteur effect. It would have been very helpful if they had identified the metabolic products that caused the drop in pH and had determined the dissolved oxygen tension in the medium. However, these results are suggestive of gradual transition from aerobic to anaerobic conditions with fermentation products appearing in the medium because of reduced oxygen availability.

A direct correlation between dissolved oxygen tension and metabolic endproducts was reported by Harrison and Pirt (89). They studied the effect of DO tension on the metabolic products of a steady state population of Klebsiella aerogenes when the growth-limiting nutrient was either glucose or nitrogen. In an excess oxygen state (DO tensions above 10 to 15 mm Hg), when growth was limited by ammonia nitrogen in the presence of excess glucose, pyruvate and small amounts of 2,3-butanediol and ethanol accumulated in the medium. In the limited oxygen state (0 to 10 mm Hg), pyruvic acid production decreased with decrease in oxygen tension, but fermentation products like butanediol, acetic

acid, ethanol, and lactate increased. Pyruvate did not accumulate when growth was glucose-limited in the presence of excess oxygen. Almost all glucose was accounted for as cells and CO_2. However, in the limited oxygen state, dry weight and CO_2 production decreased with oxygen supply, and glucose carbon appeared instead in butanediol, ethanol, and volatile acid.

2. Heterogeneous or Mixed Microbial Populations

a. Oxygen Utilization in the Activated Sludge Process

Porges, et al. (91) studied the rate of oxidation of skim milk solids as a function of dissolved oxygen concentration, and found that the rate was appreciably lower below 0.5 mg/l O_2. They recommended a mandatory maintenance of dissolved oxygen tension of 0.3 to 0.5 mg/l for the oxidative process. On the other hand, von der Emde (92) observed linear reduction of dissolved oxygen content of the medium down to zero level during the oxidation of dilute milk by activated sludge. He felt that intensive mixing was the cause for linear DO removal.

A systematic study on oxygen consumption rates of washed activated sludge suspended in phosphate buffer over a wide range of partial pressures of oxygen in the gas phase (15 to 748 mm Hg) was carried out by Wuhrmann (93) under constant mixing conditions in a Warburg apparatus. He found that the respiration rate of the sludge was not affected even by low oxygen pressures, provided the turbulence in the suspension was sufficient for the continuous dispersion of the sludge flocs. At very high sludge concentrations (18,000 mg/l), however, there was a marked increase in the respiration rate with increase in dissolved oxygen tension. The rate of utilization of glucose added to

2800 mg/l of sludge suspended in phosphate buffer remained almost the same at DO concentrations of 28.6, 7.9, and 1.3 mg/l; a slight reduction occurred at the lowest level.

Using activated sludge grown in batch culture with glucose and ammonium sulfate, van Gils (94) examined the influence of increased oxygen tension on the oxygen uptake rate of the sludge and the dissimilation percentage of glucose; the study was performed using the Warburg apparatus. At three dissolved oxygen tensions obtained by employing 20, 60, and 100 per cent oxygen in the gas phase, he did not find any effect of the increased oxygen tension on the oxygen uptake of the cells during either the substrate assimilation or endogenous phase. There was also no change in the dissimilation percentage of the substrate.

The independent effect of dissolved oxygen concentration on the oxygen uptake rate of heterogeneous populations in continuous flow steady state systems was investigated by Rickard and Gaudy (95). Under constant environmental conditions, varying the DO concentration in the range of 1.4 to 7.1 mg/l had no effect on the oxygen uptake rate of the system. They concluded that the existence of stagnation layers around the bacterial cell is not an important factor for governing O_2 uptake in well-agitated completely-mixed systems. It is significant to note that they did not observe any gross change in predominating species. The lowest DO examined in their studies (1.4 mg/l) was considerably higher than the critical DO reported for microorganisms.

b. Oxygen Tension and Purification Efficiency of the Activated Sludge Process

Several workers have studied the relation between DO in the aeration tank and the performance of the activated sludge process.

Wuhrmann (96) reported increased elimination of organic carbon or nitrogen with small or medium concentrations of sludge (600 mg/l) when DO concentration of the mixed liquor increased from 1 to 4 mg/l. He could not find any noticeable improvement of performance by a further increase of DO tension up to 7 mg/l. His data also indicated inhibition of nitrification when the DO fell to values of the order of 0.5 mg/l.

Orford, et al. (97) have observed that when the DO concentration in the mixed liquor was below 0.5 mg/l, the 5-day BOD of the settled effluents increased. Smith (85) did not find any effect of DO tension, within the range of normal operation, on the efficiency of BOD removal in continuous flow activated sludge process provided "a residual DO content existed in the mixed liquor at all times." He noted an immediate decrease in BOD removal efficiency in the absence of "measurable" DO (presumably anaerobic conditions). von der Emde (92) recommended that the DO content of the aeration tank should be at least 2 mg/l since, according to him, below this value the purification effect would decrease.

Even depletion of DO, if not prolonged, may not impair the purification capacity of the activated sludge process, according to Gaudy and Turner (86), who observed that short-term depletion of DO during shock loading of activated sludge did not severely affect the biochemical efficiency of the system. They suggested that the values of oxygen tension which affect the metabolic rate lie below 0.5 mg/l.

Rickard and Gaudy (95) observed a small improvement in both COD and glucose removal efficiency with an increase in DO concentration from 1.4 to 7.1 mg/l. Biological solids yield, cell RNA, and protein were unaffected, but there was a significant decrease in cell carbohydrate

with increase in DO.

c. Oxygen Tension and Ecology of Activated Sludge

Heukelekian (98) observed that the viable count developed in a BOD bottle varied over wide limits as the initial DO was changed. He attributed this variation to the development of different populations at different DO levels.

The increased substrate removal efficiency obtained by Wuhrmann (96) when the DO was increased from 1 to 4 mg/l was attributed to a change in the species composition of the sludge. He theorized, without direct experimental evidence, that "with the increase of oxygen tension, gradually more and more aerobic organisms dominate the usual microaerophilic and facultative aerobic species, predominant at low aeration intensities." Indirect evidence for this premise was provided by the altered characteristics of the sludge resulting from the increase in oxygen concentrations.

von der Emde (92) has also observed ecological changes in activated sludge at different oxygen tensions and BOD loadings. At high BOD loads, flagellates replaced the ciliates even at high DO levels. However, with low DO and BOD load, a decrease in the number of stalked ciliates and an increase in the free-swimming forms and flagellates was observed. Bacteria alone were present when only traces of DO were present.

Low oxygen tensions have also been implicated as a causative factor for the proliferation of Sphaerotilus (99), and hence bulking in activated sludge.

d. Oxygen Tension and Shock Loads

Krishnan and Gaudy (17) made a detailed study on the response of completely mixed activated sludge systems to gradual and slug

quantitative shock loads at several detention times, with or without solids recirculation. The experiments were carried out at high DO levels, and the DO did not undergo considerable reduction due to the introduction of shock loads. The response of the systems to shock loads was therefore not limited by the DO level in the systems .

Rickard (18) made some preliminary studies on the effect of agitation on the ability of completely-mixed systems to handle shock loads. At a velocity gradient of 300 sec^{-1} the DO dropped to nearly zero within two hours after increasing the feed concentration from 1000 to 3000 mg/l glucose at a detention time of five hours. The system showed oxygen limitation, since the O_2 uptake rate and biological solids level decreased after an initial period of increase. Glucose removal was quite complete even in the near absence of DO, but the effluent contained large amounts of metabolic intermediates and/or endproducts. No volatile acids were present, but a trace of ethanol was detected. Increasing the velocity gradient at the same level of shock load had a pronounced effect on the COD removal of the system. Leakage of COD during the early stages of shock decreased progressively with the increase in the velocity gradient. No differences in the level of glucose in the effluent were noted. However, as much as 80 per cent of the effluent COD was present as acetate. Rickard's studies suggest that mixing energy is a factor in the response of completely-mixed systems to quantitative shock loading. Since the DO concentration in the systems also varied with velocity gradient, it is possible that it may also play a role in the ability of the system to handle shock loads successfully.

3. Summary

There seems to be a unanimity of opinion that oxygen limitation

causes changes in the metabolic patterns of microorganisms. However, there are conflicting reports regarding the minimum dissolved oxygen level to be maintained in an aerobic reactor such as those used in the activated sludge process. No advantage seems to accrue by providing excess oxygen to the organisms. Available evidence suggests that the purification efficiency of the activated sludge process may be adversely affected below a DO level of about 0.5 mg/l. There is a paucity of information on the possible effect of low oxygen tensions on the ability of activated sludge systems to successfully handle quantitative shock loads.

B. Oxidative Assimilation in Microorganisms

1. General Comments

The process of oxidative assimilation of substrates can be defined as the primary conversion of the substrate to a primary product of assimilation, the raw material for all secondary synthesis within the cell (100). As early as 1936, Barker determined the oxygen utilization by Prototheca zopfii during oxidative assimilation of various compounds (100). Since the organism was known to synthesize and store glycogen, Barker concluded that glycogen was the primary product of assimilation of these substrates which might be used for secondary synthesis within the cells during conditions where growth was possible.

Even though oxidative assimilation takes place in the presence or absence of exogenous nitrogen, workers in the microbiological fields employed suspensions of cells in suitable buffers and devoid of nitrogen for studies on oxidative metabolism in many microorganisms. Most of the early studies on oxidative assimilation were conducted using monometric procedures. Clifton (101)(102) has published excellent reviews

of the early studies.

Calculations based on oxygen uptake data alone sometimes led the early workers in this field to erroneous conclusions, since in most cases it was assumed that the substrate not accounted for as oxidized was assimilated by the cells. The advent of radiotracer techniques during the last decade has enabled investigators to assess, quantitatively, the distribution of the substrates in various cellular fractions and in the supernatant. With the help of manometry and tracer techniques, it has been possible to determine more precisely the amount and nature of assimilation and the extent of oxidation.

2. Definition of Terms Used in Oxidative Assimilation Studies

The terms "respiring system," "resting cell system," and "nonproliferating system" are used interchangeably in microbiological and biochemical fields to describe suspensions of cells in a buffered medium containing the carbon source but devoid of nitrogen source. For the purpose of the present study, a "resting cell system" may be defined as suspension of the cells in a buffer containing only the carbon source, and a "nonproliferating system" as one containing all of the nutrients of the growth medium in which the cells were previously grown, but devoid of the nitrogen source. A "growing system" will be one in which all of the nutrients are provided in order to allow growth (replication).

3. Oxidative Assimilation by Pure Cultures

Although numerous studies have been carried out on the oxidative metabolism of several organisms, some selected papers will be reviewed in the following pages.

Employing washed cells of Bacillus cereus grown previously on nutrient agar containing glucose and suspended in phosphate buffer

(pH 7.2), Clifton and Sobek (103), and Clifton (104) used the Warburg apparatus to study the oxidative assimilation of uniformly labeled glucose. They found that approximately 50 per cent of the glucose carbon was assimilated by the cells, and the rest was oxidized.

Binnie, et al. (105) studied the oxidative assimilation of uniformly labeled glucose by cells of Sarcina lutea suspended in phosphate buffer (pH 7.1). The cells had been harvested from a peptone medium and had their endogenous respiration reduced to negligible levels prior to the experiment. All of the radioactivity assimilated by the cells (55 per cent of that added) was accounted for as glucose appearing as polysaccharide within the cells.

Studies by Clifton (106) on Bacillus megaterium showed that, during oxidative assimilation of glucose, a dynamic metabolic state existed within the cell rather than deposition of the storage products only, since radioactivity from the substrate appeared in all fractions of the cell. Carbohydrate did not appear to be a major assimilation product of glucose utilization. At the time of disappearance of the substrate (glucose) from the medium, 39 per cent had been oxidized, and 37 per cent assimilated. This organism accumulated considerable amounts of poly-β-hydroxy-butyrate during oxidative assimilation of glucose. Macrae and Wilkinson (107) have also reported that this organism can accumulate as much as 40 per cent of its dry weight as poly-β-hydroxybutyrate in a nitrogen-deficient medium.

Nutrient agar-grown Escherichia coli K-12 assimilated about 50 per cent of the glucose added to washed suspensions of the cells in phosphate buffer (pH 7.2)(73). However, cells grown on glucose agar assimilated only 20 to 30 per cent of exogenously supplied glucose under

similar conditions. There was no difference in the amount of glucose oxidized (about 36-37 per cent) by the two types of cells. The difference in the metabolism has been suggested as due to enzymatic differences between the cells, resulting from the nature of the growth medium on which the cells were grown prior to the experiment.

Pseudomonas aeruginosa provides an example of the fact that not all bacteria are capable of accumulating specialized storage polymers. It does not accumulate carbohydrate, lipid (including poly-β-hydroxybutyrate) or polyphosphate under oxidative assimilation conditions (69)(108). Using washed suspensions of cells grown in a synthetic medium containing glucose, Duncan and Campbell (69) found that only 10 to 15 per cent of glucose carbon was assimilated by the resting cells at the time of glucose disappearance from the medium; about 50 per cent of the glucose was oxidized. Most of the synthesized material was found to be nitrogenous, the nitrogen being supplied by endogenous metabolism which continued unabated in the presence or absence of exogenous glucose.

These studies on the oxidative assimilation of glucose by resting cells of bacteria show that the extent of assimilation of the substrate and the nature of the assimilation product depend upon the nature of the organism and its previous growth history. The substrate not accounted for by oxidation and assimilation was present in the medium as metabolic intermediates and/or endproducts. The conditions leading to their elaboration are discussed in another section of this report.

4. Oxidative Assimilation by Heterogenous Populations

Ruchhoft, et al. (109) studied the oxidation of glucose by activated sludge in the presence or absence of nitrogen, and found that the percent glucose oxidized by acclimated populations at the end of 24

hours after feeding was the same (16.8 per cent) in both cases. Placak and Ruchhoft (110) conducted classic experiments on the oxidative assimilation of thirty-six pure organic compounds by activated sludge in the presence of ammonia nitrogen. They reported that organic acids were primarily oxidized while high proportions of carbohydrates were assimilated.

Porges, et al. (111) distinguished three processes involved in the rapid removal of skim milk COD (containing lactose and casein with adequate nitrogen) by acclimated activated sludge. They were oxidation, synthesis, and storage. On the basis of their hypothetical equation for oxidative assimilation and the actual amount of oxygen utilized, they computed the COD used for cell formation and energy. By subtracting this amount from the total COD removed, they arrived at the values for COD stored. According to this computation they found that in two hours at $30^{o}C$, 1000 mg/l sludge removed 89 per cent of 1125 mg/l available COD, or 1000 mg/l. Of this, only 11 per cent was oxidized to CO_2, 18 per cent was converted to cell substance, and 70 per cent was stored. Analysis of the stored material showed that it was a polymer of glucose. Storage of a large amount of substrate in the form of glycogen resulted in cells with low nitrogen content. These investigators suggested use of an empirical formula for sludge composition. Studies involving the use of such an empirical formula should be interpreted with caution. Use of direct chemical analyses of the sludge would have yielded more accurate and reliable results on the nature and extent of storage.

Wuhrmann (93) reported similar low oxidation of carbohydrates. Using a washed suspension of unacclimated sludge in phosphate buffer, he found that only 13.5 per cent of added sucrose was oxidized and the

remainder was assimilated. Similar experiments with glucose, acetate, and butyrate indicated percent respiration of 17.1, 30.6, and 28.0, respectively. In the presence of nitrogen, however, oxidation of glucose was increased to 50 per cent.

A detailed investigation of the gross biochemical changes taking place in aerobic heterogeneous populations under growing and nonproliferating conditions was carried out by Gaudy and Engelbrecht (15). Employing glucose-acclimated activated sludge harvested from a continuous flow unit, they examined the metabolic patterns of both systems after feeding glucose; the experiments were conducted in Warburg flasks. The substrate removal and solids accumulation achieved by the system devoid of nitrogen was comparable to those under growth conditions. Both systems had identical solids levels (COD to biological solids ratio of 0.9) at the beginning of the experiment. The major difference in the biochemical composition of the two sludges was that under nonproliferating conditions carbohydrate was the major product accumulated, whereas under growing conditions protein was the major synthesis product. A greater portion of the substrate removed was channelled into synthesis in the absence of nitrogen. A significant aspect of their study was the finding that protein synthesis continued even after the exhaustion of glucose from the medium, and cellular carbohydrate served as the source for carbon skeletons for protein synthesis in the endogenous phase.

van Gils (94) observed oxidation of about 15 per cent of the glucose added to a washed suspension of activated sludge which had been grown on glucose and $(NH_4)_2SO_4$; 60 per cent was stored as polysaccharide. Addition of $(NH_4)_2SO_4$ increased the dissimilation percentage only

slightly. The nature of the assimilation product in the presence of nitrogen was, however, different; only 25 per cent of the glucose removed was converted into polysaccharides, and 50 per cent into unknown compounds. These "unknown" compounds were converted into nitrogenous compounds during the endogenous phase. He also found that initial COD to initial biological solids ratio had no effect on the percent oxidation of glucose.

RamaRao, et al. (5) made a comparative study on the performance of activated sludge, acclimated earlier to glucose, acetate, and phenol under growing and nonproliferating conditions at initial COD to solids ratios of 2, 1, and 0.5. Their results showed that all systems removed the added substrate with almost the same efficiency, although in some cases the nonproliferating systems with higher COD to solids ratio required a longer period to accomplish the same COD removal as in the corresponding growing systems.

C. Conditions Known to Influence Carbohydrate Synthesis in Microorganisms

The various conditions that lead to the accumulation of carbohydrate in microorganisms have been reviewed by Pannbacker and Wright (112). Of importance in the present study are the effects of nitrogen starvation, nature of the substrate, and food to microorganism ratio on the ability of cells to store carbohydrates.

1. Type of Substrate

a. Pure Cultures

Dagley and Johnson (113) noted a close relationship between the composition of the cellular reserves and that of the medium in growing

Escherichia coli cells. By adding glucose to cells grown in acetate medium, they showed that the level of polysaccharide rose with increasing glucose concentration in the medium, and this was accompanied by a fall in lipid level. Addition of increasing amounts of acetate to glucose-grown cells resulted in higher lipid levels and lower polysaccharide concentration.

Holme (75) found that the rate of glycogen synthesis by steady state cultures of Escherichia coli depended on the nature of the carbon source in the medium. Using nitrogen as the growth-limiting factor and glucose or lactate as the carbon source at various dilution rates, he noted that, when the rate of synthesis of nitrogen-containing compounds was high, glycogen was synthesized at a slower rate when lactate was the carbon source than when glucose was used. He attributed this difference to the fact that a longer series of transformations is required for synthesis from lactate than from glucose. This difference in the relative synthetic rates diminished when the rate of synthesis of nitrogenous compounds was reduced, thereby making available a greater portion of carbon source for glycogen synthesis.

Binnie, et al. (105) did not find a detectible increase in the carbohydrate content of resting cells of Sarcina lutea, pre-grown in a peptone medium, during oxidative assimilation of pyruvate and acetate. This led them to suggest that glycolytic reactions were not reversible under these conditions. However, assimilation of glycerol resulted in a considerable increase in carbohydrate content of the cells. They therefore concluded that the block in the reversal of glycolysis occurred between pyruvate and triosephosphate, or alternatively, that glucose was synthesized from glycerol by another pathway.

b. Heterogeneous Populations

Walters, et al. (114) studied the ability of yeast extract-glucose-acclimated cells to synthesize carbohydrate with glucose, acetic acid, or glutamic acid as substrate. Even though the cells were acclimated to the new substrates, only the cells fed glucose stored carbohydrate. It is interesting to note that the pH of the systems fed acetic and glutamic acids were 8.4 and 8.5, respectively, whereas the glucose system had a pH of 7.0. It is not known whether the higher pH had any effect on the metabolism of acetic and glutamic acid systems.

Goel and Gaudy (115) found that the immediate storage product of oxidative assimilation of acetic acid by acclimated nonproliferating populations was not carbohydrate. Gaudy and Engelbrecht (15), and Komolrit, Goel, and Gaudy (7) had shown earlier that the primary product of oxidative assimilation of glucose by nonproliferating cells was carbohydrate.

Quantitative difference in the carbohydrate content of activated sludge during the metabolism of dairy waste was noted by Adamse (116). There was an initial accumulation of carbohydrates in the cells during the rapid metabolism of the lactose portion of the wastes. When the lactose was eliminated from the medium, protein decomposition was at its maximum, and there was a decrease in the initially-accumulated polysaccharides.

2. Food to Microorganisms Ratio

Using activated sludge grown on yeast extract and glucose, Walters, et al. (114) studied the influence of the "F/M ratio" on the synthesis of cellular carbohydrate and poly-β-hydroxybutyrate (PHB). When their results were expressed as maximum percent of substrate COD

converted to carbohydrate COD, it was seen that between F/M ratios of 0.78 and 4.3 a constant maximum of 40 per cent of the substrate was converted to carbohydrate. However, beyond an F/M ratio of 4.3, carbohydrate storage rapidly decreased, indicating that more substrate was diverted to replicative pathways rather than storage.

D. Release of Metabolic Intermediates by Microorganisms

The factors influencing the secretion of extracellular metabolic products in microorganisms have been enumerated earlier in this report. An evaluation of earlier work in this field is attempted in this section.

1. Pure Cultures

a. "Resting" Cells

Clifton's studies on the oxidative assimilation of glucose by "resting" cells of Bacillus megaterium (106), Bacillus subtilis (102), and Escherichia coli (73) revealed a disturbing factor that caused complications in evaluating the partition of substrate between synthesis and oxidation. As much as 41 per cent of the C^{14} in U-C^{14}-glucose was present in the medium as materials other than glucose at the time of elimination of glucose by resting cells of Bacillus megaterium. This non-glucose material was, however, subsequently metabolized at a slow rate. Small amounts of lactic acid and pentose were detected in the medium. The nature of the bulk of the intermediates was not determined.

An interesting observation during the oxidative assimilation of glucose by resting cells of Escherichia coli was made by Clifton (73). Whereas cells grown earlier on nutrient agar released 14 per cent of the radioactivity from U-C^{14}-glucose into the medium as metabolic

intermediates, glucose agar-grown cells secreted 41 per cent of the initial glucose in the form of such products as lactic acid, acetic acid, and ethanol. On the basis of additional experiments on the production of volatile acids under stationary, shaken, and anaerobic conditions he concluded that glucose fermentation was an inherent characteristic of glucose-grown cells, although the extent of fermentation was reduced under aeration conditions. He suggested the existence of regulatory mechanisms in the cells which controlled the extent of fermentation under aerobic conditions. He did not offer any satisfactory explanation for the difference in the fermentative characteristics of nutrient agar-grown and glucose-agar-grown cells. It is also not known whether the cells suffered any O_2 limitation even under "shaken" conditions. Oxygen tension in the medium was not measured.

Duncan and Campbell (69) observed the accumulation of a large quantity of α-ketoglutarate during the rapid oxidation of glucose by washed suspensions of Pseudomonas aeruginosa in Tris buffer (pH 7.2). The cells had previously been grown in a synthetic medium containing glucose and ammonium phosphate. Maximum accumulation of α-ketoglutarate (equivalent to 35 per cent of glucose carbon) was observed at the time when glucose was eliminated from the medium. After the disappearance of glucose, α-ketoglutarate was gradually oxidized. In the presence of added ammonia, no α-ketoglutarate appeared in the medium. Since this organism was known not to store any "primary product" during oxidative assimilation, and since ammonia was released during the endogenous phase, these authors concluded that ammonia nitrogen was the limiting factor in the incorporation of α-ketoglutarate into cell material. They theorized that the presence of a partial metabolic

block at the α-ketoglutarate level of carbohydrate oxidation was advantageous to the organism, since it permitted the conservation of the carbon compound which would act as the point of entry to protein and nucleic acid syntheses upon the availability of ammonia. In support of their premise, they stated that the organism possessed a stong glutamic dehydrogenase. It is not known why the metabolic block occurs at this keto acid level only; if protein synthesis is prevented, other keto acids which also serve as points of entry to protein might also be expected to accumulate.

A large accumulation of pyruvate and α-ketoglutarate was observed by Mackelvie, et al. (117) during nitrogen-limited growth of Pseudomonas aeruginosa in a synthetic medium containing excess glucose. The authors did not give any explanation for the large accumulation of intermediates. It is interesting to note, however, that this organism did not accumulate any reserve carbohydrate or lipid under these conditions, and hence probably could not divert a part of the substrate for the synthesis of reserve material.

b. Growing Cells

Release of volatile acids like acetic and pyruvic acids during aerobic growth of Escherichia coli (73)(118), Pseudomonas natrigens (67), and Aerobacter aerogenes (119), on glucose in a mineral salts medium has been reported by other workers. Amarasingham and Davis (63) theorized that an advantage of "aerobic glycolysis" of glucose, resulting in the formation of partially-oxidized product(s) "might be derived from the transformation of part of the sugar to a product that would not be useful to certain competing organisms; such a product might then remain 'stored' in the culture fluid for future use."

2. Heterogeneous Microbial Populations

a. Type of Substrate

Adamse (116) observed the release of acid intermediates during the metabolism of lactose present in dairy wastes due to the activity of aerobic microorganisms. Acid production occurred even when the minimal DO stayed well above zero. The acid intermediates were identified as mainly acetic acid, which was subsequently utilized. He could not say whether the formation of large amounts of acetic acid was due to the presence in the sludge of a well-defined type of bacterium, or whether the specific conditions in the sludge floc were responsible.

b. Shock Loads

George and Gaudy (120) studied the effect of pH and hydraulic shock loadings on the performance of the activated sludge process. When a completely-mixed activated sludge system was subjected to a hydraulic overload (decreased detention time) with a constant substrate concentration, the effluents during the transient state contained metabolic intermediates, the amount increasing with overload. Similarly, when the system was subjected to an increase in detention time under constant organic loading conditions, the effluent contained a large amount of intermediates.

When the shock load involved a change in pH of the feed from 6.65 to 6.15, the solids level in a completely-mixed reactor decreased and then rose again. Although there was no immediate leakage of COD after the shock, there was a significant leakage at the time of the lowest solids level. Most of this COD was due to metabolic intermediates which were either diluted out or utilized during the subsequent buildup of the solids in the system. Additional experiments at different pH

levels revealed that the predominating microbial species in the reactor changed from rod-shaped bacteria before the shock to filamentous forms during the transient state.

Komolrit and Gaudy (121) observed the release of large amounts of metabolic intermediates during quantitative shock loads and combinations of quantitative and qualitative shock loads in a completely-mixed continuous flow reactor when the shock loads were accompanied by an increase in BOD:N ratio.

Krishnan and Gaudy (122) detected large amounts of metabolic intermediates during the metabolism of glucose following the shock loading of glucose to young cells acclimated to glycerol under nonproliferating conditions in a batch system. These metabolites were not re-utilized in the absence of exogenous nitrogen.

Komolrit and Gaudy (121) studied the responses of a completely-mixed steady state system growing on sorbitol when it was subjected to qualitative shock loads (sorbitol + glucose) with or without a concomitant increase in organic loading. The effluent after the qualitative shock contained large amounts of intermediates which increased when the shock load was accompanied by an increase in organic loading. They stated that the "effect of glucose on the utilization of either sorbitol or glycerol at relatively low combined qualitative and quantitative shock load ratios was primarily evidenced by a release of large concentrations of metabolic intermediates."

The response of lysine-degradative systems in heterogeneous populations to a qualitative shock load with carbohydrates under batch conditions was found by Grady and Gaudy (123) to be a function of the growth rates on, and production of metabolic intermediates from, those

carbohydrates. Since glucose supported relatively rapid growth with the production of many metabolic intermediates, it had a relatively greater effect upon the production of lysine-degrading enzymes. An increase in the rate of formation of "enzymatic capability" for the lysine-degrading system did not occur until the level of these intermediates in the medium had been reduced. These findings were confirmed in continuous flow steady state systems.

Krishnan and Gaudy (17) observed the release of metabolic intermediates during gradual quantitative shock loading of continuous flow steady state systems using glucose. The amount of intermediates was found to be dependent upon the detention time, extent of shock load, BOD:N ratio in the shock feed, and whether the solids were recirculated. The lower the detention time, the greater was the production of intermediates after the shock loading. Experiments employing solids recirculation indicated very little accumulation of intermediates even when the shock load was accompanied by a higher BOD:N ratio. The major intermediate produced during shock loading without cell recyle was acetic acid, although smaller amounts of other free fatty acids were also secreted. Under nitrogen-deficient conditions the accumulated intermediates were not utilized, indicating that the enzymes required for the utilization of these intermediates were inducible and nitrogen was required for their synthesis.

3. Summary

Studies on pure cultures reveal that accumulation of partially oxidized compounds can occur even during balanced growth on substrates like glucose. This situation is accentuated in the absence of an essential nutrient like nitrogen. Information on the metabolism of

compounds other than glucose under conditions known to stimulate the release of intermediates is scant. Studies with heterogeneous populations subjected to different types of shock loads indicate that the amount and nature of the shock have profound influence on the release of metabolic intermediates during the transient phase. Under shock load conditions, metabolism of compounds which are utilized for growth more rapidly than is the substrate to which the cells have been acclimated can lead to a large extracellular accumulation of intermediates.

E. Studies on the Endogenous Oxidation of Cell Components

1. Pure Cultures

Ribbons and Dawes (124) have shown that Escherichia coli grown on a glucose-ammonium salts medium always contained glycogen, and on subsequent starvation in buffer solution this glycogen was rapidly metabolized. The preferential utilization of glycogen prevented the net degradation of nitrogenous materials. However, tryptone-grown cells which were always devoid of glycogen, released ammonia immediately upon starvation.

The work of Strange, et al. (125)(126), and Postgate and Hunter (36)(39) reveals that washed suspensions of Aerobacter aerogenes harvested from carbon-limiting mannitol-ammonium salts medium contained very little carbohydrate and degraded their protein without any change in carbohydrate content. However, the cells harvested from tryptone-glucose medium had a high carbohydrate content, and during the initial 25 hours of starvation glycogen was depleted from the cells, accompanied by a small change in protein and no change in RNA content.

Using cells of Sarcina lutea harvested from a peptone medium, Binnie, et al. (105) studied the endogenous utilization of cellular

carbohydrate accumulated during incubation with glucose in the absence of nitrogen. The cells were allowed to assimilate glucose for two hours; they were then washed, resuspended in phosphate buffer, and gently aerated for forty-eight hours. Ultimately all of the assimilated carbohydrate was utilized, although the rate of its disappearance was much less in the later stages. A high concentration of cells (600 mg cells and 2 mM glucose in 20 ml suspensions) was used in the assimilation experiment. It is not known whether prolonging the aeration beyond forty-eight hours would have resulted in a further decrease in cellular carbohydrate. However, earlier experiments (127) had shown that the "structural polysaccharide" content of the peptone-grown cells was about 10 per cent and did not undergo a detectible decrease during the endogenous phase. Dawes and Ribbons (128) pointed out that the only compounds now shown not to be utilized as endogenous substrates are DNA, constituents of cell walls, and membranes and extracellular slime layers.

2. Heterogeneous Microbial Populations

Hoover, et al. (129) found that the average endogenous respiration rate for cells fed skim milk solids was 10 µl O_2/hr/mg cells. Using the equation for the endogenous oxidation of the sludge whose chemical formula had been previously established, they calculated that the cell tissue was oxidized endogenously at the rate of one per cent per hour, and hence a system containing 2500 mg/l sludge when fed 1000 mg/l skim milk solids (which will produce 500 mg/l sludge) would not result in the accumulation of solids if the detention time is twenty hours. They suggested the possibility of total oxidation of the sludge under these conditions. In a later communication (91) they stated that the

complete oxidation of 500 mg/l cells by autoxidative digestion would require about 160 hours.

Forney and Kountz (130) supported the theory of "total oxidation" and provided evidence for the establishment of solids equilibrium in a continuous flow system treating skim milk wastes. Symons and McKinney (10), however, rejected the theory of "total oxidation." They operated a batch-fed activated sludge system on sodium acetate with 100 per cent sludge recycle for a period of thirty-five days and found that the systems at all COD:N levels accumulated volatile biological solids during the entire period of operation. There was a buildup of a material, identified by staining as extracellular polysaccharide, which was resistant to degradation during prolonged endogenous respiration. They concluded that batch-fed or conventional activated sludge systems cannot operate without sludge wasting.

Kountz and Forney (11) later conceded that total endogenous oxidation was not possible "within reasonable times," since there accumulated a residual material in a continuously fed activated sludge system operated by them for six months, which amounted to 20 to 25 per cent by weight of the new sludge produced.

Washington and Symons (13) studied the accumulation of volatile solids in batch-fed and completely-mixed continuous flow activated sludge systems grown on glucose, sodium acetate, or glycine. The accumulation of volatile solids amounted to about 10 to 15 per cent of the ultimate BOD of the substrate removed under equilibrium operations for wastes which were carbohydrate or fatty acid in nature. They also conducted studies on the extent of degradation of various cellular components of the sludge from the three systems under endogenous conditions

for twenty-seven days. The protein and fat fractions were found to be readily degradable during the endogenous respiration phase. The carbohydrate content of the sludge increased with the time of autolysis, indicating the greater extent of inertness in the carbohydrate fraction of the cells. They theorized that the biologically inert volatile solids which would be expected to accumulate in the activated sludge system would average from 47 to 56 per cent polysaccharide, 39 to 47 per cent protein, and 3 to 8 per cent fats.

Changes in cell weight, oxygen uptake, supernatant COD, and ammonia nitrogen during an extended period of aeration (twenty-five days) of a once-fed batch activated sludge system were investigated by McWhorter and Heukelekian (14). The decrease in cell mass during the first day of endogenous respiration was 25 to 33 per cent of the maximum existing at the time of glucose removal. The rate of oxidation of cell mass approached zero in ten to twelve days. An inactive cell mass which remained after twenty-five days of aeration amounted to 40 per cent of the maximum sludge mass produced, or 12 per cent of the theoretical oxygen demand of the glucose fed. They did not identify the nature of the inactive cell mass; however, its low nitrogen content indicated to them that the inactive portion was carbonaceous material which was not readily utilized. They also found that the residual COD amounted to from 5 to 15 per cent of the initial substrate COD. The changes in the nitrogen content of the cell mass during substrate removal and endogenous phases suggested to these workers that storage material was produced during the substrate removal phase and that it was subsequenly utilized in the early endogenous phase. They did not perform any direct analysis for cellular carbohydrate.

F. Release of Extracellular Metabolic Products During Endogenous Metabolism

Liberation of ammonia into the medium during the endogenous respiration of several bacteria has been reported (69)(108)(117)(131)(132)(133). The sources of the ammonia have been identified as cell protein and RNA. These studies also showed that either exogenously added glucose or an internal carbohydrate store had a sparing action on the breakdown of nitrogenous constituents during starvation.

Degradation products of RNA have also been observed in the medium during the endogenous phase in many organisms (28)(75)(125)(126)(128)(132). Clifton (132) observed that as much as 70 per cent of radioactivity from uracil-labeled cells was secreted into the medium during ninety-six hours of endogenous metabolism in Escherichia coli K-12.

Most of the studies on pure cultures were performed for short periods of time under starvation conditions, and indicate the lability of the cellular constituents when exogenous food supply is exhausted. Release of ammonia nitrogen during endogenous oxidation of activated sludge has also been observed by McWhorter and Heukelekian (14).

CHAPTER IV

MATERIALS AND METHODS

A. General Conditions

1. Batch Experiments

a. Heterogeneous Microbial Populations

An initial seed of heterogeneous populations was obtained from the primary clarifier effluent of the municipal treatment plant of Stillwater, Oklahoma.

b. Growth Medium

The composition of the synthetic waste used as the growth medium in these studies is given in Table I. Whenever higher substrate concentrations were used, the concentrations of other constituents were correspondingly increased. The composition of the growth medium was so chosen that the carbon and energy source limits growth under conditions of otherwise adequate nutrition.

c. Growth Conditions

The organisms were fed the synthetic waste medium in a batch reactor of 1.5-liter capacity and aerated for twenty-three hours. Compressed air at a pressure of 30 psi was supplied in excess by means of sintered glass diffusers. After twenty-three hours of aeration, one-third of the mixed liquor was wasted and the remainder was settled for

TABLE I

COMPOSITION OF THE GROWTH MEDIUM

Component	Concentration
Substrate	1000 mg/l
$(NH_4)_2SO_4$	500 mg/l
$MgSO_4 \cdot 7\ H_2O$	100 mg/l
$MnSO_4 \cdot H_2O$	10 mg/l
$CaCl_2$	7.5 mg/l
$FeCl_3 \cdot 6\ H_2O$	0.5 mg/l
Tap Water	100 ml/l
1.0 M Potassium Phosphate Buffer (pH 7.0)	10 ml/l
Distilled Water	To Volume

one hour. Following the settling period, one-half of the remaining volume was wasted, and the mixed liquor was made up to the 1.5-liter mark with the waste medium. The heterogeneous populations were thus acclimated to each substrate for a period of not less than four weeks, and checked for solids balance and COD removal. After the attainment of solids balance and constant COD removal, feeding was continued for several days prior to initiation of any experiments.

Dissolved oxygen measurements in the reactor during growth of the organisms indicated a high residual (4 to 6 mg/l) even during active growth on all substrates used. The reactor was maintained in a constant temperature room, and the temperature of the mixed liquor varied between 22.5 and 24.5^{o}C. The pH registered a small drop (a few tenths of a unit) during growth on all substrates except fatty acids and sodium acetate when an increase was noted.

2. Continuous Flow Experiments

a. Experimental Reactor

For the continuous flow experiments a 5-liter Microferm laboratory fermenter (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey) was used. This was converted to a single stage continuous flow reactor by the addition of an influent feed line and an inverted standpipe, positive suction effluent line. Pumping of the feed to and effluent from the reactor was accomplished by peristaltic pumps (Sigmamotor Model AL-4E). A schematic diagram of the reactor is shown in Figure 2.

b. Experimental Conditions

The aerating gas, filtered through glass wool, was passed through

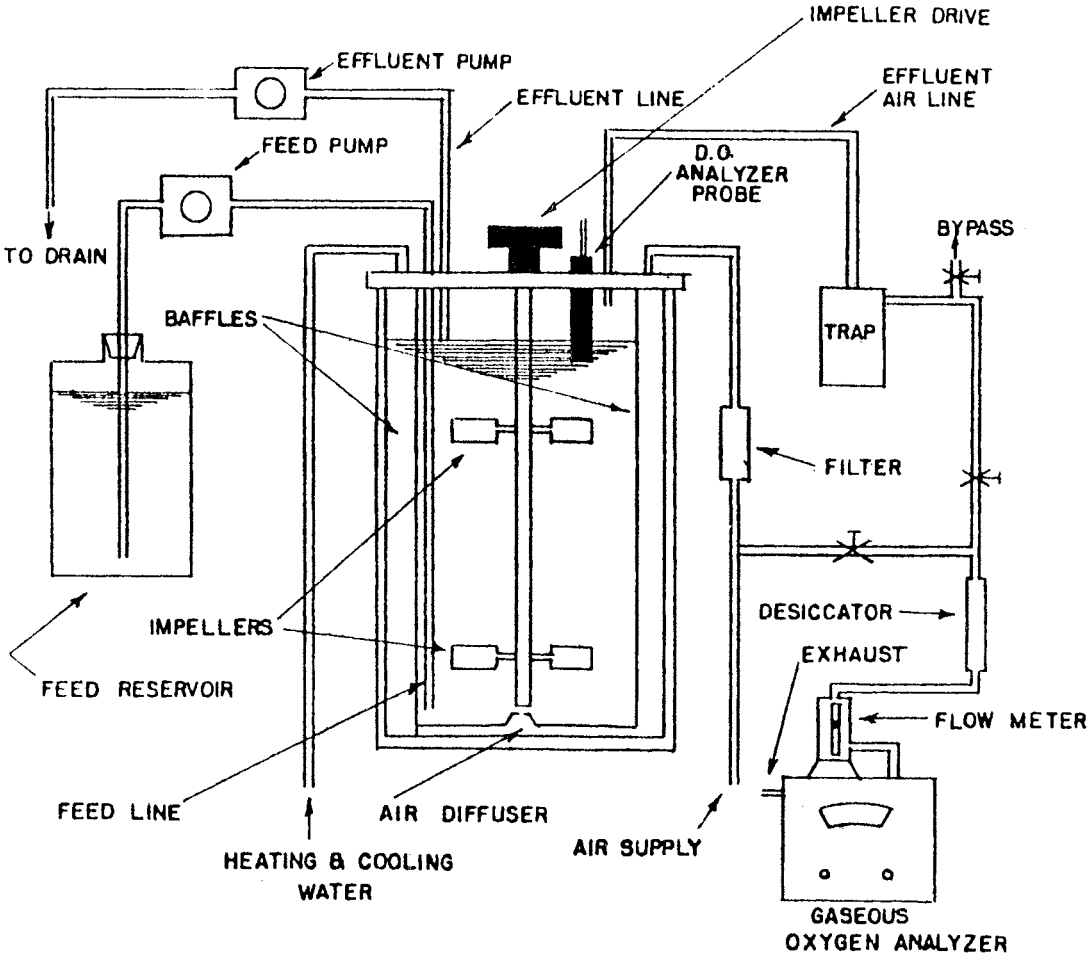


Figure 2 - Schematic diagram of reactor employed for continuous flow experiments.

the single air outlet in the bottom of the reactor at the rate of one liter/min. The temperature of the reactor was controlled at 30 ± 0.25° C. The speed of the impellers was set at 300 rpm. The velocity (mixing) gradient provided to the medium at these agitation and airflow rates was found to be 680 sec^{-1} (50). A detention time of eight hours (i.e., D = 0.125 hr^{-1}) was employed. These experimental conditions were kept constant throughout the period of this study.

The effective volume of the medium in the reactor at the above-mentioned velocity gradient was experimentally determined. This was done by filling the reactor with a measured volume of water, setting the agitation and gas flow rate at the required level, pumping and measuring the excess water above the standpipe level. The effective volume was then determined by subtraction. It was found to be 3810 ml. The required flowrate to give a detention time of eight hours was 476 ml/hr. The feed and effluent lines were changed periodically and cleaned by pumping Clorox solution and distilled water through them.

c. Heterogeneous Microbial Populations and Growth Medium

The source of the initial seed of heterogeneous populations and the growth medium employed in continuous flow studies were the same as those used for batch experiments. Glucose was the substrate used.

d. "Steady State" Operation

The reactor was filled with the growth medium and 200 ml of the sewage seed, and operated as a batch system for twenty-four hours to develop an ample microbial population. Feed was then pumped at a rate of 476 ml/hr, and the reactor was operated under continuous flow conditions for several detention times. The reactor was then checked for

"steady state" operation by determining the concentrations of biological solids, dissolved oxygen, and filtrate COD of the mixed liquor in the reactor as well as the oxygen uptake rate and pH of the reactor. Relative constancy of these parameters during several detention times was taken as an indication of "steady state." Steady state operation was then continued for several detention times before initiating the shock load experiments. Whenever wall growth was noticed, the reactor was lowered from the head plate, the sides of the wall were scraped with a nylon brush, and the reactor was replaced.

At least four samples were taken for each "steady state" during the operation at different DO concentrations. The sampling period covered at least three detention times during each "steady state." The average value of each parameter was taken as being representative of the "steady state" value.

B. Experimental Protocol

1. Short-term Batch Experiments

The organisms were harvested by centrifuging from the stock reactor twenty-three hours after the previous feeding, and washed twice in the growth medium devoid of the substrate and $(NH_4)_2SO_4$. The washed cells were blended for ten seconds in a Waring blender, and suspended in the washing medium. The substrate was then added to give the desired initial concentration. This suspension was divided into two portions, and to one portion $(NH_4)_2SO_4$ was added. The portion of the suspension which contained all of the nutrients in excess but a limiting amount of the carbon source will hereinafter be referred to as the "growing system." The other portion of the suspension which contained all of the nutrients of the growth medium except nitrogen will be

referred to as the "nonproliferating system."

Initial samples were withdrawn from each system after thorough mixing, and aeration was then begun. Conditions of batch growth employed were the same as explained previously. Dissolved oxygen and pH were monitored throughout the course of all experiments. Samples of the mixed liquor were withdrawn periodically during the experiment.

Oxygen uptake data were obtained for some experiments using the Warburg respirometer run concurrently with the batch reactors. Forty ml portions of the suspension of each system were placed in 140 ml Warburg flasks before starting the aeration in the batch reactors. The Warburg flasks contained 1.5 ml of 20 per cent KOH in the center well. The apparatus was run at $25^{o} \pm 0.5^{o}C$ with a shaker rate of 110 oscillations per minute. Endogenous oxygen uptake under growing and nonproliferating conditions was also determined using 40 ml cell suspensions devoid of the substrate. Glucose, sucrose, lactose, sodium acetate, acetic and propionic acids were employed as substrates.

2. Long-term Batch Experiments

The experimental protocol was the same as for short-term experiments except for the procedure adopted to avoid water loss by evaporation during prolonged aeration. The long-term batch experiments were performed in four-liter Erlenmeyer flasks with two sintered glass diffusers in each to provide adequate mixing. Compressed air was saturated with water by bubbling it through distilled water prior to its entry to the flasks. Beginning on the second day of the experiment, the volume of the mixed liquor was measured before each sampling, and the evaporation loss was made up with distilled water. Care was taken not to lose any solids during this operation. Glycerol and

sorbitol were used as substrates for this study.

3. Continuous Flow Experiments

a. Effect of Different Dissolved Oxygen Tensions

Steady state behavior of the system operating with 1000 mg/l glucose as the growth-limiting substance were studied at different dissolved oxygen tensions obtained by changing the oxygen content in the gas supply. The gas mixtures employed were air, 10 per cent O_2 + 90 per cent N_2, and 5 per cent O_2 + 95 per cent N_2.

b. Shock Loading Experiments

The shock load studies involved doubling or tripling the concentration of glucose in the feed of the "steady state" systems operating at various DO levels. Samples of the mixed liquor were withdrawn immediately prior to the initiation of the shock loads, during the transient phase, and during the new steady state at the higher substrate concentration. Responses of the steady state systems at the higher and lower steady state DO concentrations during gradual withdrawal of the shock load were also investigated. These experimental conditions allowed the study of transient responses of the systems after an increase or decrease in the concentration of the growth-limiting nutrient in the feed, and also facilitated a comparative study of the steady state systems over a wide range of DO levels.

4. Sampling Procedures for Batch and Continuous Flow Experiments

Two 40 ml samples of the mixed liquor were collected from the reactors at each sampling period, and were immediately centrifuged and filtered. Immediate analyses were performed for the total COD of the filtrate and for the biological solids of one 40 ml sample of the mixed

liquor. The solids collected from the other sample and the remaining filtrates were frozen immediately and stored at -15° for later analysis. The collection of the above solids sample for cell composition analysis was according to the method outlined by Gaudy (134). Additional 40 ml samples of mixed liquor were also collected for lipid analysis in some batch experiments.

C. Methods of Analysis

The analytical methods employed were the same for both batch and continuous flow experiments except where noted. All colorimetric determinations were performed with a Bausch and Lomb Spectronic 20 colorimeter/spectrophotometer with 1/2-inch matched test tubes.

1. Biological Solids

The biological solids concentration of the mixed liquor was determined by filtration through membrane filters (0.45 μ pore size, Millipore Filter Corp., Bedford, Mass.) according to Standard Methods (135).

2. Chemical Oxygen Demand

The total COD of the membrane filtrate was determined in accordance with Standard Methods (135). Mercuric sulfate and silver sulfate were used for all COD determinations. The COD of the feed samples for continuous flow experiments was also determined at each sampling.

3. Glucose

For concentrations of glucose above 50 mg/l in the filtrate, the enzymatic Glucostat test (Worthington Biochemical Corp., Freehold, N. J.) (136) was adopted.

4. Total Carbohydrate - Anthrone Test

a. Membrane Filtrate

The membrane filtrates of the mixed liquor samples which had COD values less than 50 mg/l were analyzed for total carbohydrates by the anthrone method (134).

b. Biological Solids

The solids suspension was homogenized by sonic oscillation, and an aliquot of the homogenized suspension was analyzed for total carbohydrates by the anthrone method using glucose as the standard.

5. Protein Content of Biological Solids

a. The Biuret Test

The protein content of the homogenized solids suspension was estimated by the Biuret procedure as outlined by Gaudy (134). This method was adopted for a range of concentrations of 1 to 10 mg protein in the sludge sample, the maximum volume taken for analysis being 2.5 ml. Bovine plasma albumin, Cryst., A grade (Calbiochem, Los Angeles, Calif.) was used as the standard.

b. The Folin-Ciocalteau Method

For concentrations of protein below the range for the Biuret test, the Folin-Ciocalteau method (137) was employed. Aliquots of sludge samples containing 0.1 to 0.6 mg protein were analyzed by this method. The protein standard used for the Biuret test was employed.

6. Lipid Content of Biological Solids

Lipid content of the biological solids was determined by the method reported by Kincannon (138). Lipids were extracted from the biological solids for six hours using an ethanol-ether mixture on a shaker

apparatus at 25°C. The extract was then filtered through a membrane filter. The filtrate was quantitatively transferred into a COD flask, and the solvent mixture was evaporated off at 70°C. The residue left in the flasks was then flushed with a gentle air stream. The COD of the material left in the flask was determined according to Standard Methods (135). Blanks and standards containing different amounts of stearic acid were also given the same treatment as the experimental samples after the extraction procedure. Lipids were reported as equivalent stearic acid.

7. Sorbitol and Glycerol

Sorbitol and glycerol in the membrane filtrate were determined by the periodate oxidation method of Neish (139) with an oxidation time of ten minutes to allow a more complete oxidation (140).

8. Ribose

The concentration of ribose in the membrane filtrates was measured by the orcinol test (137).

9. Fructose

Free fructose in the membrane filtrate was determined by the resorcinol method of Roe for ketohexoses (141). This method gives the same molar extinction for free and esterified fructose.

10. Sucrose

In the batch experiments employing sucrose as the substrate it was found that sucrose was hydrolyzed into the monosaccharides very rapidly in the first few hours of the experiment. Several colorimetric methods and their modifications for the determination of total carbohydrates, reducing sugars, and ketohexoses were tried in order to estimate the

individual sugars (sucrose, glucose, and fructose) in the mixture. The following indirect method of arriving at the concentrations of each sugar was found to be satisfactory:

a. Free Glucose

Free glucose in the membrane filtrate was determined by the Glucostat procedure.

b. Free Fructose

The total absorbance contributed by both free glucose and free fructose in the Nelson test for reducing sugars (137) was determined in an aliquot of the filtrate. Both glucose and fructose standards were included in the reducing sugar determination, since it was found that fructose had a slightly higher reducing power in the Nelson test than glucose. Since the concentration of free glucose in the sample was known, the portion of the absorbance due to free fructose was calculated by difference. The concentration of free fructose was then determined using the standard curve for fructose.

c. Combined Fructose

The conditions employed in the resorcinol method (141) for ketohexoses resulted in complete hydrolysis of sucrose, and hence the method measured the combined fructose as well as the free fructose present in the filtrate. Therefore this method was adopted for measuring the total ketoses in the sample. Fructose was used as the standard.

The amount of combined fructose (present as sucrose) was obtained by subtracting the value for free fructose from that of total ketohexose.

d. Sucrose

Since one mole of combined fructose represented one mole of sucrose, the concentration of the latter could be computed.

11. Lactose

When lactose was used as the substrate, the membrane filtrate was analyzed for lactose by the anthrone test. Lactose was used as the standard. Since this disaccharide was not found to be hydrolyzed extracellularly by the organisms, this procedure was adopted. However, small amounts of galactose were present in the medium at the beginning of the experiments, possibly as an impurity in the lactose used.

12. Galactose

Galactose in the membrane filtrate of the lactose systems was determined colorimetrically by the Galactostat method (136).

13. Total Keto-acids and Pyruvic Acid

Total keto acids in the membrane filtrate were determined by the method of Friedeman and Haugen (142) as modified by Hamilton, et al. (143). The method consists of adding one ml of 2, 4-dinitrophenyl-hydrazine to 3 ml of the sample kept previously at 25^{o}C for ten minutes, incubating the mixture at 25^{o}C for twenty-five minutes and developing the color by the addition of 5 ml of 2N NaOH. The optical density was then measured at 540 mμ. Pyruvic acid was used as the standard.

Pyruvic acid was measured by reducing the incubation time after the addition of the hydrazine reagent, from twenty-five to five minutes and measuring the optical density at 520 mμ. This procedure is reported to be highly specific for pyruvic acid (142).

14. Analysis of Lower Free Fatty Acids by Gas Liquid Chromatography

a. Apparatus and Conditions

Gas liquid chromatography (GLC) was employed in this study as a rapid and reliable method for the identification and quantitation of lower free fatty acids present in the membrane filtrates. The apparatus used was a Model 810 gas chromatograph (F and M Scientific Division, Hewlett-Packard, Avondale, Pa.) equipped with hydrogen flame detectors and a Minneapolis-Honeywell recorder.

A precoiled column of 3/16-inch inside diameter packed with a porous polymer, "PolyPak-2" of 80-120 mesh size (Hewlett-Packard, Avondale, Pa.), which was thermally stable at 300°C in an oxygen-free atmosphere was used throughout these studies. The column was preconditioned before use by heating overnight at 200°C with a flow of helium which was employed as the carrier gas. It was operated isothermally during all analyses. The following analytical conditions were employed for the determination of lower free fatty acids:

Helium flow rate @ 60 psi	50 ml/min
Hydrogen flow rate @ 10 psi	28 ml/min
Airflow rate @ 20 psi	295 ml/min
Column temperature	190°C
Flame detector temperature	230°C
Injection port temperature	225°C
Volume of sample injected	5 µl

When a sample containing a mixture of lower free fatty acids was injected into the column, the acids were separated and eluted in the following order: acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids.

b. Analyses of Membrane Filtrates by GLC

The filtrates of mixed liquor samples from experiments reported in this study were analyzed by GLC for the following purposes:

i) Quantitative estimation of acetate and propionate which were used as substrates in some batch experiments

ii) Identification and quantitation of lower free fatty acids present in the membrane filtrates as metabolic intermediates.

The individual acids present in the experimental samples were identified by their detention times, and quantitated on the basis of the peak areas relative to those for known concentrations of respective standard acids. The concentrations of standard acids employed were in the same range as those in the samples. The range and attenuation of the instrument were suitably varied in order to obtain an optimum peak area that could be easily and accurately measured with the aid of a planimeter.

c. Linearity of Response and Quantitative Results

When the same volume of standard acids (5 µl) at different concentrations were injected into the column, the response as measured by the peak areas was found to be linear. When the peak areas were obtained at different attenuation settings and at a fixed range, then a plot of the peak area x attenuation against the concentrations of the standard acids gave a straight line. The linearity of response of acetic and propionic acids is shown in Figure 3. Similar responses were also obtained at lower concentrations of these acids. These calibration curves were used to calculate the concentrations of the acids in the unknown samples.

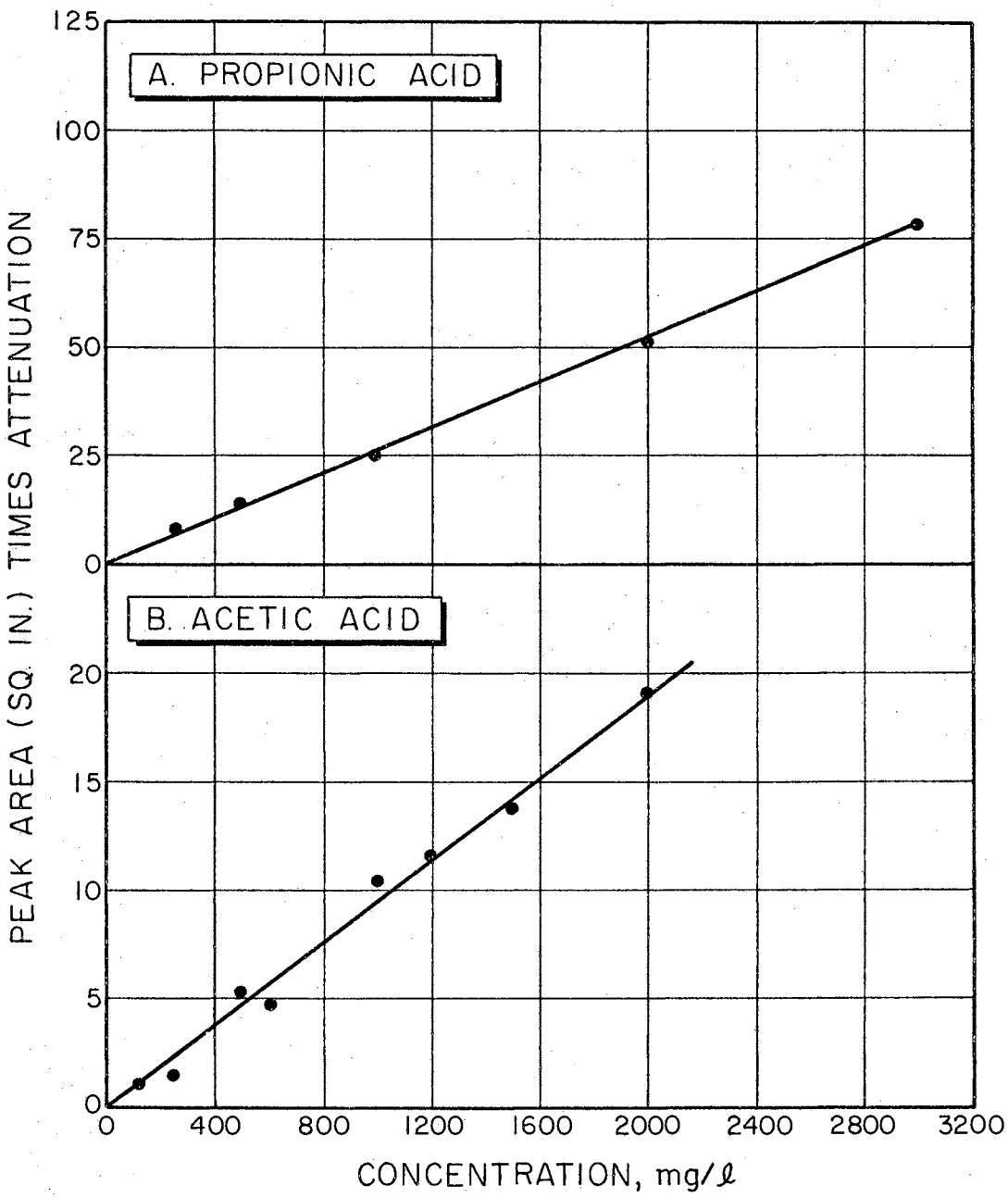


Figure 3 - Linearity of response of acetic and propionic acids in GLC analysis.

15. Nitrogen Determinations in Membrane Filtrates

a. Total Nitrogen

Five ml aliquots of the filtrate were taken in 30 ml micro-Kjeldahl flasks to each of which 5 ml of acid-sulfate solution (135) was added. The mixture was digested by boiling in a digestion apparatus for thirty minutes after the solution became clear. The mixture was then quantitatively transferred to the micro-Kjeldahl apparatus, made alkaline with hydroxide-thiosulfate solution (135), and distilled into 0.01N H_2SO_4. The excess acid was back-titrated with standard NaOH using a mixture of methyl red-methylene blue as indicator (135).

b. Ammonia Nitrogen

Five ml aliquots of the filtrates were placed directly in the Kjeldahl apparatus. Twenty-five ml phosphate buffer solution (135) were then added, and the ammonia was distilled into 0.01N H_2SO_4 as for total nitrogen determination.

c. Organic Nitrogen

The organic nitrogen content of the filtrate was calculated as the difference between the values obtained for total nitrogen and ammonia nitrogen.

16. Dissolved Oxygen

Dissolved oxygen concentrations in the experimental reactors were measured by the electrometric technique using a galvanic cell oxygen analyzer (Precision Scientific Co., Chicago, Ill.), which had a sensitivity of ± 0.1 mg/l from 5^{o} to 35^{o}C. The analyzer, which had a low zero current, was calibrated by means of the azide modification of the Winkler method for dissolved oxygen determination (135). The

sensitivity coefficient of the probe at the temperature of the sample was obtained from the chart provided by the manufacturer.

17. Oxygen Uptake Rate

Oxygen uptake rates in continuous flow studies were determined with the aid of a Beckman Model E2 gaseous oxygen analyzer. The oxygen content of the effluent gas stream was estimated by using this analyzer which had a precision of 0.01 per cent in the range used. The difference in percent oxygen contents of the influent and effluent gases was converted to milligrams of oxygen consumed per hour. This value, when divided by the volume of the medium in the reactor, gave the rate of oxygen uptake in mg/l/hr. A model calculation is shown in Appendix A. The rate of oxygen uptake per unit mass of organisms or respiration rate was also calculated as mg O_2/hr/gm dry weight.

18. Temperature and pH

The temperature and pH of the mixed liquor in the reactor were recorded at each sampling.

19. Microscopic Examination of Mixed Liquor

Samples of the mixed liquor for continuous flow experiments were examined under the microscope (wet mount as well as Gram-stained) to detect changes in the predominant populations at each stage of the experiment.

D. Expression of Results

The concentrations of the substrates, metabolic intermediates, and endproducts in the membrane filtrates were reported in terms of equivalent COD values. The theoretical COD values of the compounds employed in this study are given in Appendix B. The difference between

the total COD and substrate COD of the membrane filtrate was taken as a gross measure of the amount of metabolic products released by the cells during the substrate removal period.

CHAPTER V

RESULTS

A. Continuous Flow Experiments

The general experimental plan included establishment of a steady state at a specific level of dissolved oxygen, an increase of the concentration of substrate in the feed, observation of the transient and new steady state behavior and, in some experiments, reduction of substrate concentration to the original level and observation of the transient phase and new steady state. The experimental plan for each experiment is given in Table II. Experiments I and II were run at high DO levels, experiment III at an intermediate level, and experiment IV at a low level of DO.

Materials balances and average steady state data for the different systems before and after applying the shocks and after reducing the organic loading to its original level are presented in Table III. Materials balance calculations were made according to the method reported by Gaudy and Engelbrecht (15). Observations on the changes in microbial predominance in response to the shocks are summarized in Table IV.

1. Experiment I. Metabolic Response of the Steady State System Operating at High DO Level to a Gradual Doubling of Organic Loading

Figure 4 shows the steady state behavior of the system operating

TABLE II

EXPERIMENTAL OUTLINE FOR STUDIES ON THE EFFECT OF DISSOLVED OXYGEN LEVEL

EXPERIMENT NO.	AERATING GAS	STEADY STATE DO mg/ℓ	SHOCK FEED CONCENTRATION mg/ℓ *	PHASES STUDIED AFTER APPLYING THE SHOCK LOAD	WITHDRAWAL OF THE SHOCK	PHASES STUDIED AFTER WITHDRAWAL OF THE SHOCK
I	AIR	7.2	2000	1. TRANSIENT 2. STEADY STATE	YES	1. TRANSIENT 2. STEADY STATE
II	AIR	7.4	3000	1. TRANSIENT 2. STEADY STATE	—	—
III	10%, O_2 + 90%, N_2	3.2	2000	1. TRANSIENT	—	—
IV	5%, O_2 + 95%, N_2	1.3	2000	1. TRANSIENT 2. STEADY STATE	YES	1. TRANSIENT 2. STEADY STATE

*Pre-shock concentration of feed was 1000 mg/l glucose.

TABLE III

MATERIALS BALANCE AND AVERAGE STEADY STATE DATA OF SYSTEMS AT DIFFERENT DISSOLVED OXYGEN CONCENTRATIONS

Exp. No.	Steady State Before or After Shock	Steady State DO mg/l	pH	COD, mg/l Influent	COD, mg/l Effluent	COD Consumed mg/hr (1) mg/l	COD Consumed mg/hr (1) (as Glucose)	Percent Substrate Removed COD	Percent Substrate Removed Glucose	O_2 Uptake Rate mg/hr mg/l	O_2 Uptake Rate mg/hr (as Glucose)	Biological Solids Synthesized mg/l	Biological Solids Synthesized (1) mg/hr	Total Substrate Accounted for mg/hr (as Glucose)	Substrate Recovery Percent	Solids Yield (2) Percent	Respiration Rate mg O_2/hr/gm Dry Wt.	Percent Substrate Respired (3)	NH_4^+-N Consumed mg/l	COD Removed / NH_4^+-N Consumed	Solids Produced / NH_4^+-N Consumed	Cell Composition Percent Dry Wt. Protein	Cell Composition Percent Dry Wt. Carbohydrate
I	Before	7.2	6.60	1030	51	979	443	95	98	62	223	374	180	403	91	38	166	50	54	18	6.9	42	24
	After	6.6	6.55	1950	32	1918	868	98	100	98	351	960	461	812	94	50	102	40	90	21	10.7	36	14
	After Removal of Shock	7.4	6.59	1220	58	1162	526	95	100	64	229	460	221	450	86	40	139	43	48	24	9.6	40	19
II	Before	7.4	6.65	1120	86	1034	468	92	99	62	223	378	181	404	86	37	164	47	63	17	6.0	51	23
	After	5.7	6.49	3150	198	2952	1337	94	99	146	524	1308	629	1153	86	44	112	39	146	20	9.0	49	25
III	Before	3.2	6.60	1060	75	985	446	93	99	66	237	370	178	415	93	38	178	53	52	19	7.1	47	19
IV	Before	1.3	6.50	1160	91	1069	484	92	98	56	201	343	165	366	76	32	163	42	54	20	6.4	50	25
	After	0.4	6.50	2130	144	1986	899	93	98	104	373	746	358	731	81	38	139	42	93	21	8.0	46	26
	After Removal of Shock	0.7	6.70	1050	124	926	419	88	96	49	176	367	176	352	84	40	134	42	48	19	7.6	36	34

(1) Rate of COD consumption or solids growth = [influent concentration, mg/l - effluent concentration mg/l] [flow rate of 0.476 l/hr]

(2) Percent solids yield = [COD consumed, mg/l/solids synthesized, mg/l] 100

(3) Percent substrate respired = [O_2 uptake (as glucose), mg/hr/COD consumed (as glucose), mg/hr] 100.

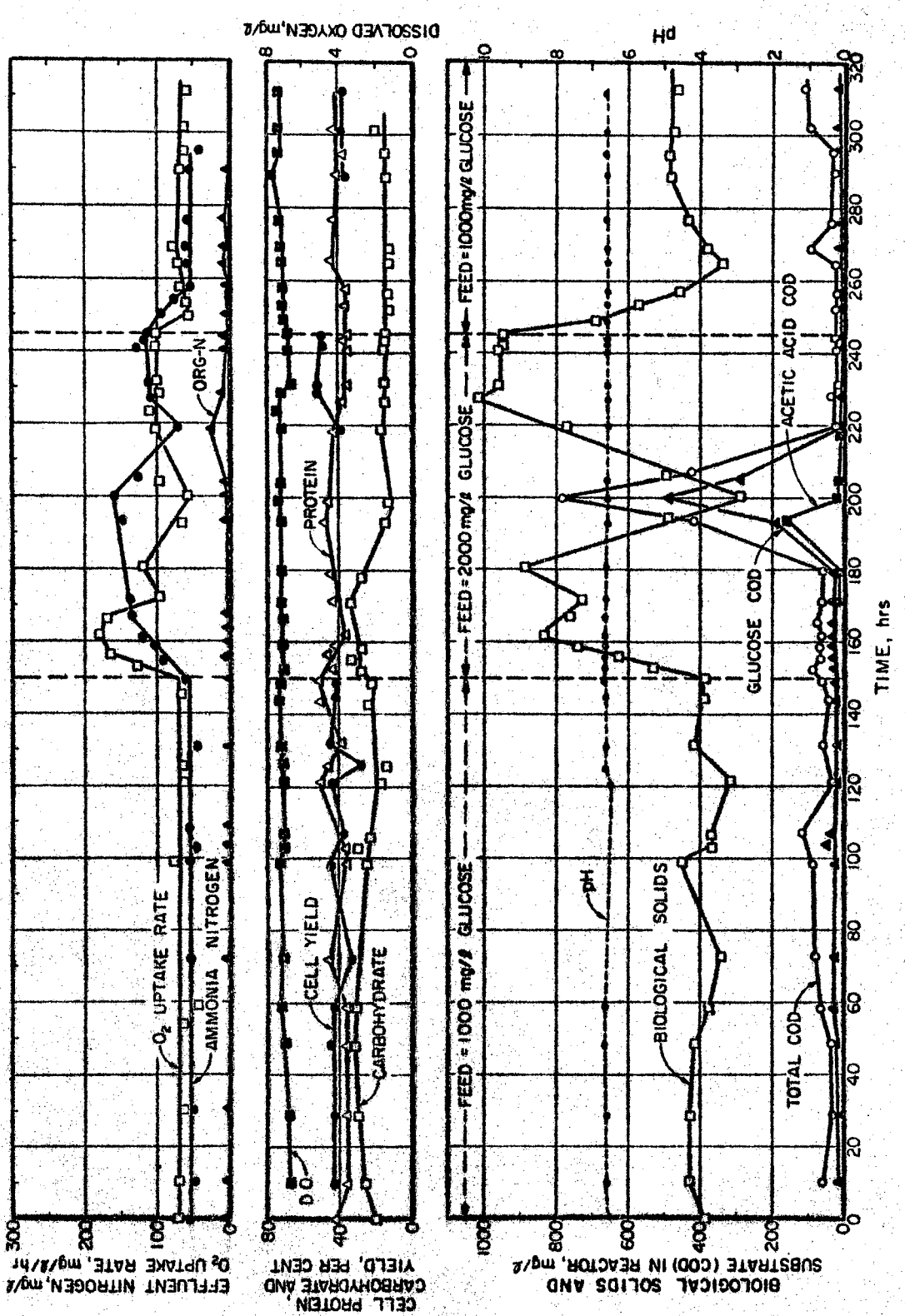
TABLE IV

MICROSCOPIC OBSERVATIONS

Exp. No.	Steady State Before Shock		Transition Phase	Steady State after Shock		Steady State after Removal of Shock	
	DO mg/l	Predominating Population	Predominating Population	DO mg/l	Predominating Population	DO mg/l	Predominating Population
I	7.2	Gram-negative long, curved rods	Gram-negative rods, also appearance of some filamentous forms	6.6	Mostly very small Gram-negative rods. Increasing numbers of Gram-positive filaments	7.4	Gram-positive filamentous organisms with many large Gram-negative rods
II	7.4	Gram-negative pleomorphic rods	Gram-negative rods of varying shapes and sizes	5.7	long chains of Gram-negative rods and a few cocci	-	-
III	3.2	Gram-negative rods of varying shapes and sizes	Gram-negative rods of varying shapes and sizes	-	-	-	-
IV	1.3	very small Gram-negative rods	Gram-negative rods	0.4	mostly short and thick Gram-negative rods and some filaments	0.7	Gram-negative rods and Arthrobacter-like coccoids

Note: Protozoa were present in all samples.

Figure 4 - Biological response of a completely mixed system operating at a high DO level to a two-fold increase in organic loading.


BIOLOGICAL SOLIDS AND SUBSTRATE (COD) IN REACTOR, mg/ℓ
pH
DISSOLVED OXYGEN, mg/ℓ
FEED = 1000 mg/ℓ GLUCOSE
FEED = 2000 mg/ℓ GLUCOSE
FEED = 1000 mg/ℓ GLUCOSE
TOTAL COD
BIOLOGICAL SOLIDS
pH
GLUCOSE COD
ACETIC ACID COD
TIME, hrs
CELL PROTEIN AND CARBOHYDRATE YIELD, PER CENT
DO
CELL YIELD
PROTEIN
CARBOHYDRATE
EFFLUENT NITROGEN, mg/ℓ
O2 UPTAKE RATE, mg/ℓ/hr
O2 UPTAKE RATE
AMMONIA NITROGEN
ORG-N

at an average DO concentration of 7.2 mg/l with 1030 mg/l glucose COD in the influent feed. It also shows the changes in parameters measured after introduction of the shock load of 1950 mg/l glucose COD as well as withdrawal of the shock.

During the period before the substrate concentration was changed, the system approximated a steady state with respect to the major parameters, i.e., biological solids concentration, effluent COD, oxygen uptake rate, pH, and ammonia nitrogen utilization. The average COD removal efficiency was 95 per cent during this period. The cell yield was 38 per cent. Three per cent of the influent COD was converted into metabolic intermediates and endproducts. Table V shows the maximum and minimum amounts of various intermediates detected in the system. Occasionally small amounts of acetic acid (5 to 10 mg/l) and organic nitrogen (4 to 9 mg/l) were detected in the medium. Less than 2 mg/l of keto acids were present in the reactor at any time during the steady state operation. No fructose could be detected. Only 50 per cent of the ammonia nitrogen supplied was consumed by the cells and hence the excess was left unutilized in the medium. Fifty per cent of the glucose was oxidized by the cells.

The predominating microbial population consisted of Gram-negative, long, curved rods.

The shock loading was initiated after 149 hours of steady state operation. It is seen from Figure 4 that a successful response was developed in the system during the first 30 hours after the shock was applied; the rapid increase in biological solids and the concomitant increases in rates of O_2 uptake and COD removal were sufficient to prevent a rise in effluent COD. During this period, no gross changes in

TABLE V

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING STEADY STATE OPERATION AT DIFFERENT DO LEVELS

Exp. No.	Steady State Before or After Shock	Steady State DO mg/l	Acetic Acid		Total Keto Acids		Ribose		Organic Nitrogen	
			Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
I	Before	7.2	nil	10	nil	2	-	-	nil	9
	After	6.6	trace	11	"	2	-	-	"	9
	After Removal of Shock	7.4	"	11	"	2	-	-	"	5
II	Before	7.4	nil	25	"	nil	2	3	"	nil
	After	5.7	"	nil	"	3	3	8	"	9
III	Before	3.2	"	"	trace	trace	trace	2	"	11
IV	Before	1.3	trace	26	4	5	2	3	"	12
	After	0.4	nil	56	5	6	6	10	"	12
	After Removal of Shock	0.7	trace	trace	2	2	9	15	"	16

Fructose was absent in all samples.
Concentrations are given as mg/l.

microbial predominance were evidenced; the Gram-negative rods which were predominant prior to the shock persisted, and the response appeared to be an increased assimilation of carbon source and growth of the indigenous population, i.e., an en masse biochemical or physiological response to the existing population.

However, the system had not attained a steady state, since approximately 40 hours (five detention times) after application of the shock there was a rapid decrease in biological solids concentration (70 per cent of the existing solids) and the effluent COD rose to nearly 800 mg/l. A large portion of the effluent COD was due to metabolic intermediates (glucose COD rose only to 485 mg/l). It is important to note that glucose concentration was determined both by the Glucostat method (specific for glucose) and by the anthrone test (specific for carbohydrate, i.e., polyhydroxy aldehydes and ketones). Both values are not plotted because, in general, they were the same except during times when small amounts of ribose and fructose appeared in the system. However, for the Glucostat test there was some variability in values of replicate determinations at glucose levels below 50 mg/l. Therefore, in plotting the concentrations of glucose COD, the results of the Glucostat test were used when the glucose levels in the system were above 50 mg/l, and the results of the anthrone test were used when the glucose concentration was below 50 mg/l. The point to be delineated is that the concentrations of glucose COD shown during the secondary response to the shock load represent specifically the levels of glucose which appeared in the effluent, and they are not attributable to metabolic intermediates and/or endproducts of glucose dissimilation.

The major extracellular product detected was acetic acid, which

was released by the cells beginning at about ten hours after the shock and peaked just before the maximum disruption in the utilization of glucose. It was, however, reduced to a low level (17 mg/l) at the time of maximum decrease in the biological solids level and appearance of large amounts of unused substrate. Transitory accumulations of small amounts of ribose (11 to 16 mg/l), fructose (5 to 14 mg/l), and keto acids (2 to 6 mg/l) were also noted during this period. During the phase of recovery of the solids level (200 to 220 hours), the organic nitrogen content of the effluent increased from 21 to 27 mg/l.

The biological solids concentration rose at approximately the same rate as it had declined, and the substrate removal efficiency recovered. In fact, a somewhat higher COD removal (98 per cent), and glucose removal (100 per cent) were observed after the establishment of the new steady state. A significant decrease in respiration and nitrogen consumption rates of the new steady state population was noted. Cell yield registered an increase of 12 per cent. There was a marked reduction in the carbohydrate content of the cells. Metabolic intermediates and/or endproducts in the system accounted for only two per cent of the feed COD. As in the steady state prior to the shock, small amounts of acetic acid (8 to 11 mg/l), and keto acid (2 mg/l) were detected in the medium.

There was only a slight decrease in the dissolved oxygen concentration due to the introduction of the shock load. No difference was noted in the steady state pH of the system.

The "secondary" response to the change in organic loading which involved a somewhat drastic reduction and subsequent recovery of the biological solids level in the system was characterized by a

significant change in the predominating organisms. Gram-positive filamentous forms began to appear in the system prior to the reduction in solids level, and continued to increase during the recovery phase. In the new steady state, many such organisms were observed in addition to the predominant population which was mostly small Gram-negative rods.

Figure 4 also shows the behavior of the system after decreasing the feed concentration to 1220 mg/l glucose COD. The biological solids decreased to a low level in response to the decreased food resource, and increased slightly again to establish a new steady state. In general, the transition to the lower concentration of available substrate was smooth and efficient, i.e., it progressed without any disruption of the treatment efficiency.

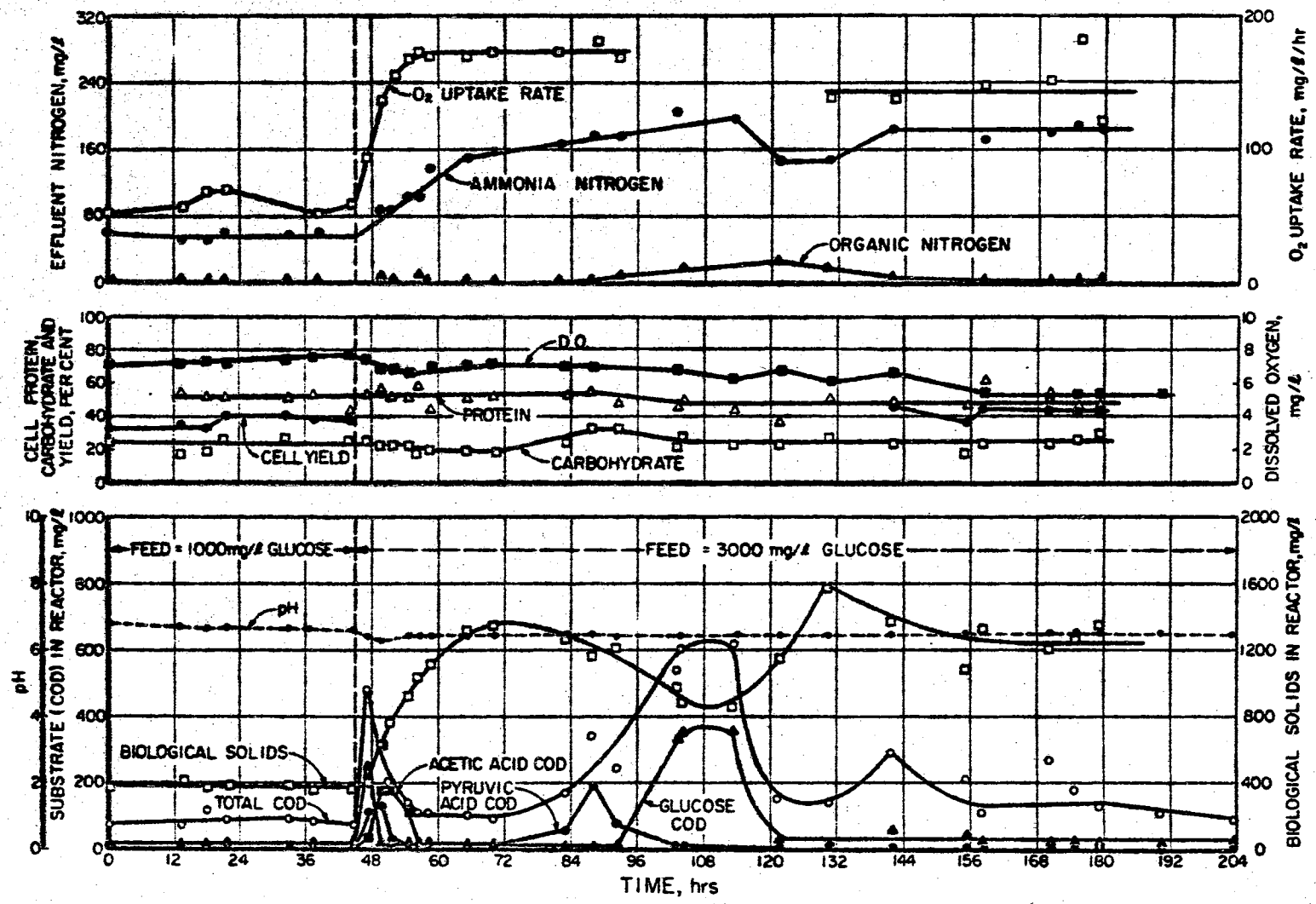
In the final steady state, the large Gram-negative rods had again increased in numbers, but many Gram-positive filamentous forms were also present. There was a slight increase in the protein and carbohydrate content of the cells. A significant increase in the respiration rate was also noted. Solids yield was lowered almost to the preshock level. The DO concentration increased slightly.

Five per cent of the influent COD was present as metabolic intermediates and/or endproducts in the reactor in the final steady state. Small amounts of acetic and keto acids and organic nitrogen were present (Table V).

2. Experiment II. Metabolic Response of the Steady State System Operating at a High DO Level to a Gradual Tripling of Organic Loading

Figure 5 shows the steady state behavior of the system operating at an average DO concentration of 7.4 mg/l with 1120 mg/l glucose COD as influent feed, and also the response of the system to an increase

Figure 5 - Biological response of a completely mixed system operating at a high DO level to a three-fold increase in organic loading.


EFFLUENT NITROGEN, mg/ℓ
O2 UPTAKE RATE
AMMONIA NITROGEN
ORGANIC NITROGEN
O2 UPTAKE RATE, mg/ℓ/hr
CELL, PROTEIN, CARBOHYDRATE AND YIELD, PER CENT
DO
PROTEIN
CELL YIELD
CARBOHYDRATE
DISSOLVED OXYGEN, mg/ℓ
pH
SUBSTRATE (COD) IN REACTOR, mg/ℓ
FEED = 1000mg/ℓ GLUCOSE
FEED = 3000 mg/ℓ GLUCOSE
pH
BIOLOGICAL SOLIDS
TOTAL COD
ACETIC ACID COD
PYRUVIC ACID COD
GLUCOSE COD
BIOLOGICAL SOLIDS IN REACTOR, mg/ℓ
TIME, hrs

in glucose COD to 3150 mg/l.

The values of the various parameters in the initial steady state were close to those observed in the previous experiment. Of the seven samples taken during the steady state operation, acetic acid was present in two in the amounts of 8 and 25 mg/l. An average of seven per cent of the influent COD was converted into metabolic intermediates and/or endproducts. No keto acids, fructose, or organic nitrogen were detected. However, 2 to 3 mg/l of ribose were present in all samples. The predominant population consisted of Gram-negative pleomorphic rods.

The higher shock load applied in this system caused a rapid rise in effluent COD. Two hours after the shock the total COD of the effluent was 475 mg/l. About 30 per cent of this COD was due to the release of pyruvic (105 mg/l) and acetic (32 mg/l) acids. Small amounts of ribose (11 mg/l) and fructose (4 mg/l) were also detected. However, the major portion (50 per cent) of this COD was due to unutilized glucose. Recovery of glucose removal in the subsequent period was rapid, but the acids persisted in the system much longer.

There appeared to be a slight "lag" in the biological solids growth at the time of maximum COD leakage. Within twelve hours after application of the shock, the system regained its COD removal efficiency and appeared to enter a new steady state which accommodated the higher organic loading. However, as in the previous experiment, there followed a secondary response in which the biological solids concentration declined and severe leakage of COD ensued. Pyruvic acid was the major contributor to the effluent COD during the initial phase of decline in solids. This was followed, however, by the appearance of large amounts of glucose in the effluent. During this secondary response there was

a significant rise in the organic nitrogen (an increase from 4 mg/l to 28 mg/l), and ribose (from 2 mg/l to 19 mg/l) in the filtrate, indicative of cell lysis. The system eventually recovered its substrate removal efficiency.

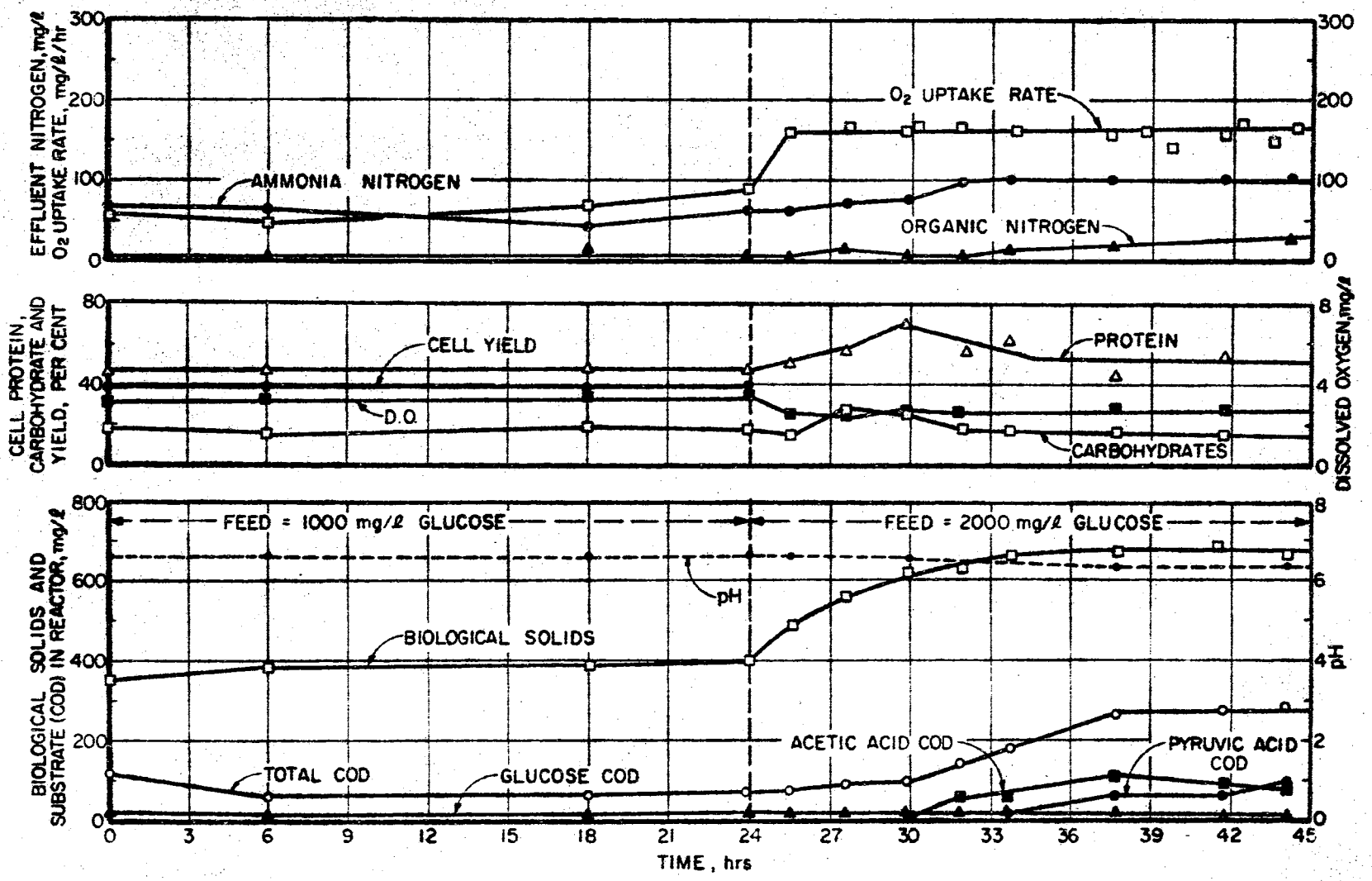
During the secondary response there was (as before) a gross change in microbial predominance. The Gram-negative pleomorphic rods, which predominated prior to the shock and throughout the primary transient response, decreased in numbers and were replaced by Gram-negative rods which grew in long chains.

Comparison of the steady state parameters before and after the shock shows a substantial increase in solids yield and decrease in substrate respiration, respiration rate, and nitrogen utilization. Five per cent of the influent COD was present as metabolic intermediates and endproducts in the new steady state, and the average DO concentration was 5.7 mg/l. The average steady state pH was slightly lower (6.5) than prior to the shock (6.7). No acetic acid was detected in the medium after the establishment of the steady state at the higher feed concentration. Ribose (3 to 8 mg/l) was present in the medium throughout the steady state operation. Small amounts of organic nitrogen were detected in a few samples.

3. Experiment III. Metabolic Response of the Steady State System Operating at an Intermediate DO Level to a Gradual Doubling of Organic Loading

The steady state behavior of the system operating at a DO level of 3.2 mg/l and the changes in the parameters measured in response to an increase in organic loading from 1060 to 2200 mg/l glucose COD are shown in Figure 6.

Figure 6 - Biological response of a completely mixed system operating at an intermediate DO level to a two-fold increase in organic loading.


EFFLUENT NITROGEN, mg/ℓ
O2 UPTAKE RATE, mg/ℓ/hr
AMMONIA NITROGEN
O2 UPTAKE RATE
ORGANIC NITROGEN
CELL PROTEIN, CARBOHYDRATE AND YIELD, PER CENT
CELL YIELD
D.O.
PROTEIN
CARBOHYDRATES
DISSOLVED OXYGEN, mg/ℓ
BIOLOGICAL SOLIDS AND SUBSTRATE (COD) IN REACTOR, mg/ℓ
FEED = 1000 mg/ℓ GLUCOSE
FEED = 2000 mg/ℓ GLUCOSE
pH
BIOLOGICAL SOLIDS
TOTAL COD
GLUCOSE COD
ACETIC ACID COD
PYRUVIC ACID COD
TIME, hrs

The values of the steady state parameters prior to the shock were essentially the same as those measured for Experiments I and II at the high DO levels, 7.2-7.4 mg/l. About six per cent of the substrate COD was present as metabolic intermediates or endproducts in the effluent. No acidic intermediates were detected in the medium during steady state operation. Trace amounts of ribose were present in the system. The predominating population consisted of Gram-negative rods of varying sizes and shapes.

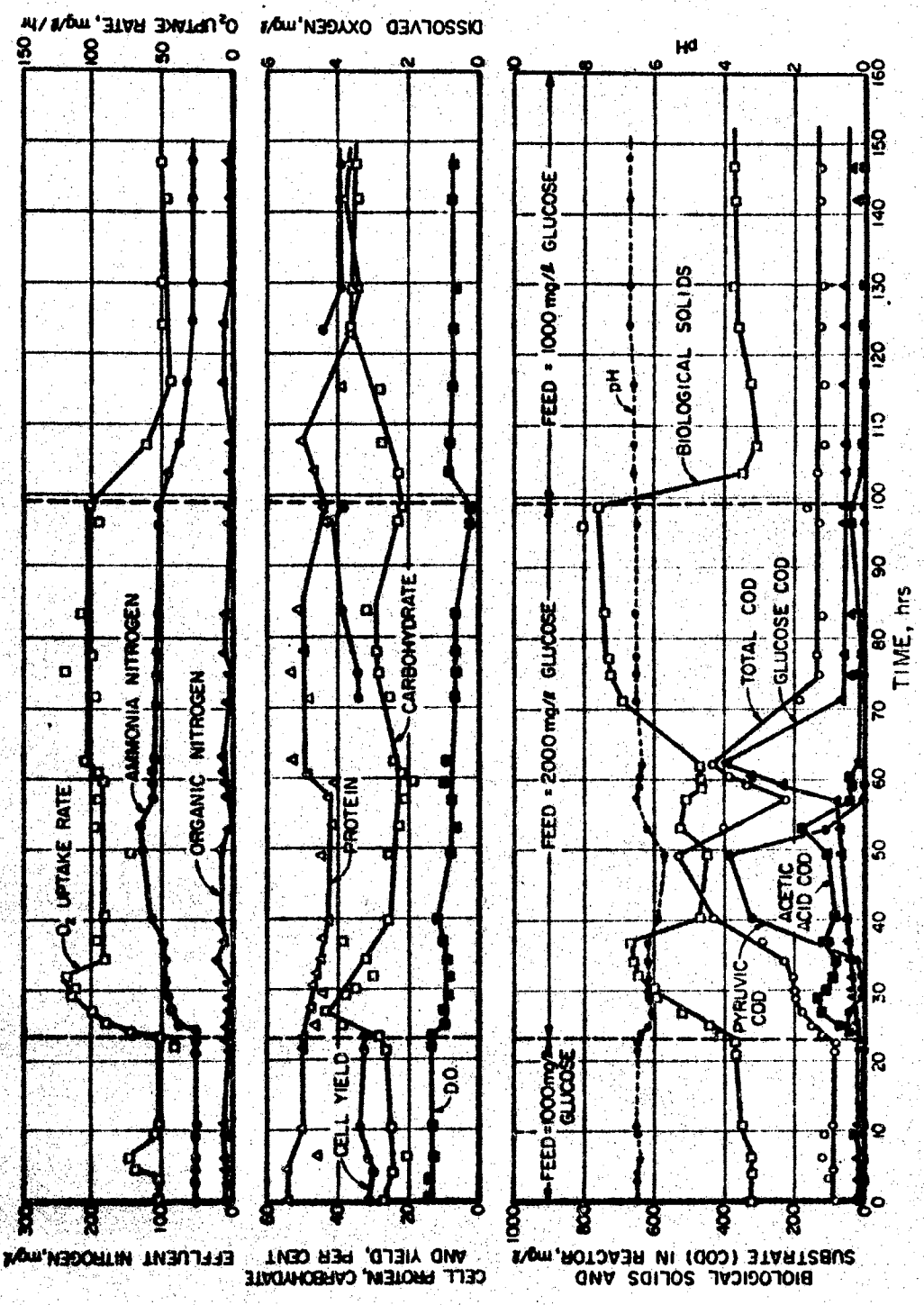
After application of the shock, the O_2 uptake rose; the DO concentration remained above 2.5 mg/l. There was no leakage of glucose, but the effluent COD rose slightly during the first six hours after the shock. During this period the biological solids concentration rose from 400 to slightly over 600 mg/l. Up to this point the immediate response was similar to that shown for Experiment I; however, the biological solids did not continue to rise rapidly, and with the slowing down of solids production there was a gradual rise in effluent COD which could be accounted for mainly as acetic and pyruvic acids. The pH of the medium decreased to 6.4 from a pre-shock value of 6.6.

Throughout the transient phase the organic nitrogen content of the filtrate increased from 3 to 22 mg/l; also some ribose was found in the effluent. Thus the somewhat sluggish solids response may have been due to lysis of a portion of the population. No change in the predominating types of microorganisms occurred during the period of observation.

4. Experiment IV. Metabolic Response of the Steady State System Operating at Low DO Level to a Gradual Doubling of Organic Loading

Figure 7 shows the response which occurred when a system growing at a feed concentration of 1100 mg/l glucose COD and a DO level of

Figure 7 - Biological response of a completely mixed system operating at a low DO level to a two-fold increase in organic loading.


BIOLOGICAL SOLIDS AND SUBSTRATE (COD) IN REACTOR, mg/ℓ
pH
FEED = 1000 mg/ℓ GLUCOSE
FEED = 2000 mg/ℓ GLUCOSE
FEED=1000mg/ℓ GLUCOSE
BIOLOGICAL SOLIDS
pH
TOTAL COD
GLUCOSE COD
ACETIC ACID COD
PYRUVIC COD
TIME, hrs
CELL PROTEIN, CARBOHYDRATE AND YIELD, PER CENT
DISSOLVED OXYGEN, mg/ℓ
CELL YIELD
PROTEIN
CARBOHYDRATE
D.O
EFFLUENT NITROGEN, mg/ℓ
O_2 UPTAKE RATE, mg/ℓ/hr
O_2 UPTAKE RATE
AMMONIA NITROGEN
ORGANIC NITROGEN

1.3 mg/l was shocked by increasing the feed concentration to the 2130 mg/l level.

In the steady state before the shock was applied, the COD removal efficiency and other system parameters were comparable to those in the system operating at the high DO levels; sludge yield was somewhat lower (Table III).

Acetic acid (up to 26 mg/l) was detected in a few samples during the steady state operation. An average of 4 mg/l of keto acids and 2 mg/l of ribose was present in all samples. About 5 to 12 mg/l of organic nitrogen were detected in the filtrate of most of the samples.

When the feed concentration was increased, there was an increase in biological solids concentration and in oxygen uptake rate; also, there was a significant increase in carbohydrate content of the sludge. There was also an increase in effluent COD concentration, due largely to accumulation of acetic acid. The acetic acid persisted in the effluent, and as the effluent COD continued to rise, large amounts of pyruvic acid appeared in the effluent. The acids were not readily metabolized, and the biological solids level decreased and stabilized at a concentration somewhat higher than the previous steady state level. During this period, large amounts of organic nitrogen (up to 24 mg/l) were detected in the filtrate.

During the period of decreasing COD concentration (49 to 57 hours) the level of pyruvic acid decreased. This was followed by a secondary transient increase in effluent COD, which involved leakage of the original carbon source (glucose). The amount of ribose in the filtrate increased to a maximum of 14 mg/l during this period.

In the initial steady state and in the early transient state,

small Gram-negative rods had been predominant. However, when the efficiency of COD removal and the biological solids concentration had increased, the microbial population consisted predominantly of short thick rods and some filaments.

The average DO level in the reactor decreased by a few tenths of one mg/l after the shock loading. It remained, however, above 0.6 mg/l throughout the transient phase. The pH of the medium decreased progressively after application of the shock. At the time of maximum production of pyruvic acid, it reached the minimum level of 5.7. It subsequently rose again to stabilize at the new steady state value of 6.5, which was the same as the average pH of the system prior to the shock loading.

The average DO level in the steady state after the shock loading was 0.4 mg/l. There was practically no change in the COD removal efficiency of the steady state system at this lower level of DO. An average of five per cent of the influent COD was converted into metabolic intermediates and endproducts. Random variations in the acetic acid content of the filtrate were noted; the concentration remained between zero and 15 mg/l during most of the period. However, somewhat higher amounts (50 mg/l) were present in two samples during the latter part of steady state operation. Small amounts of ribose and keto acids were also present. About 50 per cent of the samples contained 7 to 12 mg/l organic nitrogen.

There was an appreciable decrease in the respiration rate, and an increase in solids yield in the steady state at the higher organic loading.

There was no deleterious effect on the system during transition

to the decreased concentration of glucose in the feed. The average DO after the steady state was reached was 0.7 mg/l. In the new steady state there was a slight reduction (5 per cent) in the COD removal efficiency of the system.

A somewhat larger portion (8 per cent) of the initial COD was transformed into metabolic intermediates and/or endproducts. Only trace amounts of acetic and keto acids were detected in the system. However, some ribose (9 to 15 mg/l) persisted in the system throughout the steady state operation. The filtrate of one sample taken during the initial steady state operation contained 16 mg/l organic nitrogen. Subsequent samples, however, did not show any significant amount.

In the new steady state, many Arthobacter-like coccoids were also present in the system along with Gram-negative rods.

B. Short-term Batch Studies on the Metabolism of Carbohydrates and Fatty Acids Under Growing and Nonproliferating Conditions

1. Metabolism of Glucose

The experimental plan included the study of the response of glucose-acclimated cells under growing and nonproliferating conditions at three different initial COD:biological solids ratios, i.e., 7.5, 2.8, and 1.3. The cells employed in this study were harvested from the same stock reactor, which had been acclimated to 2000 mg/l of the substrate.

a. Experiment I. Metabolism of Glucose by Growing and Nonproliferating Cells at High Initial COD:Biological Solids Ratio

Figure 8 shows the response of the growing and nonproliferating systems when an initial COD:biological solids ratio of 7.5 was employed

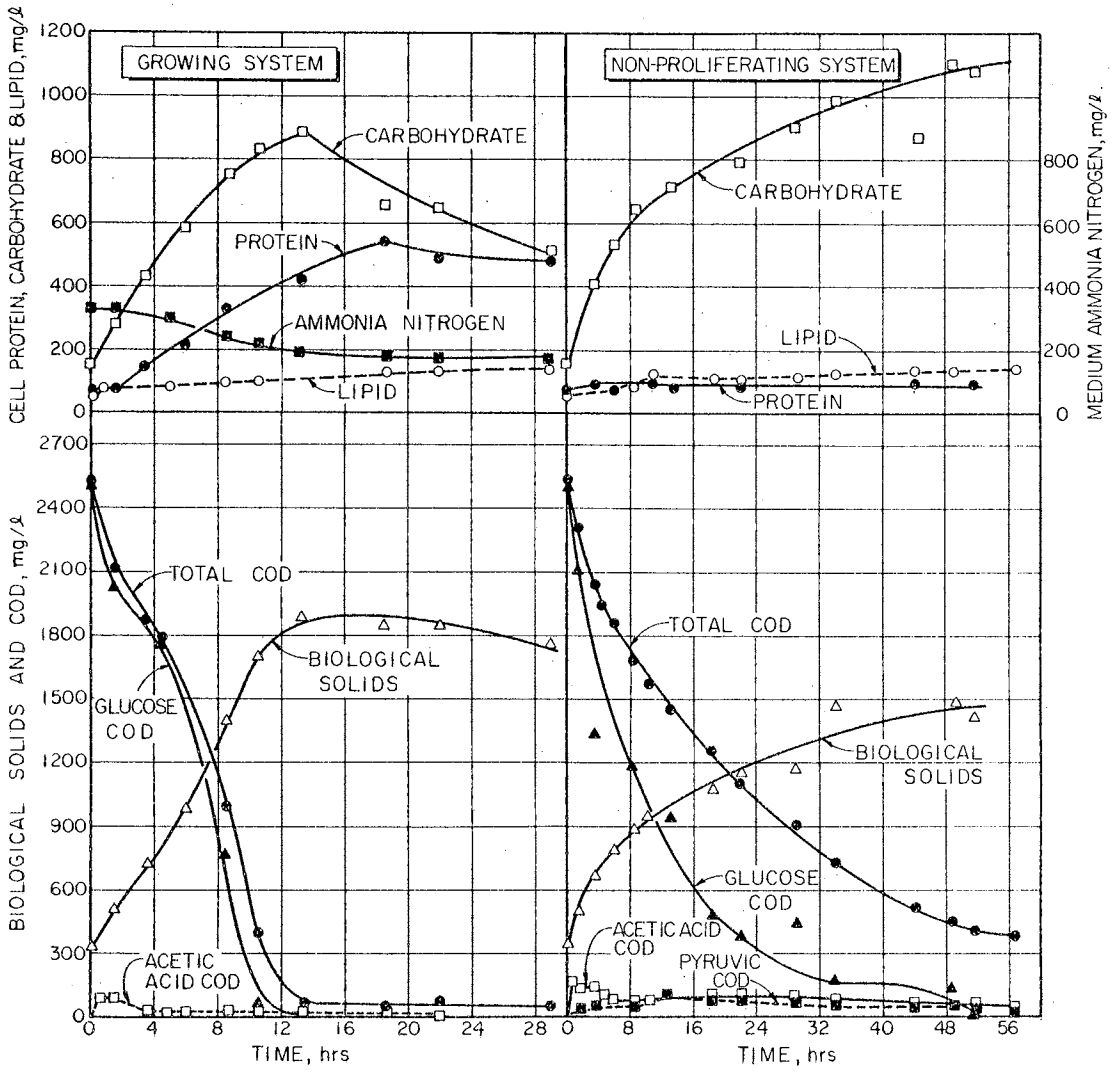


Figure 8 - Response of growing and nonproliferating systems during metabolism of glucose at high initial COD:biological solids ratio (Experiment I).

Whereas COD removal and solids growth were exponential (first order, increasing rate) in the growing system, COD removal followed decreasing first order kinetics under nonproliferating conditions (Figure 9). Ninety-seven per cent of the initial COD was removed within thirteen hours of aeration in the growing system. In the system devoid of exogenous nitrogen, 84 per cent of the initial COD was stabilized at the end of 52 hours. Cell yield was slightly lower in the latter system.

A significant difference between the two systems was the rate of removal of glucose COD. Glucose was removed much faster than total COD in the nonproliferating system, and consequently a large amount of metabolic intermediates and/or endproducts accumulated in this system. At the end of about 19 hours of aeration, as much as 31 per cent of the initial COD was converted to non-glucose COD. Only about 50 per cent of these intermediary compounds was subsequently metabolized. The residual COD at the termination of the experiment (57 hours) amounted to 16 per cent of the initial COD and was composed entirely of metabolic endproducts.

Table VI shows the amounts of metabolic intermediates and endproducts at various times during the course of the experiment. It is seen that although acetic and pyruvic acids were formed under both conditions of operation, the amounts released were relatively larger in the nonproliferating system. It is interesting to note that the production of acetic acid was highest during the initial period of glucose metabolism in both systems. Elaboration of large amounts of pyruvic acid followed the release of acetic acid in the nonproliferating system. While both acids were subsequently metabolized in the

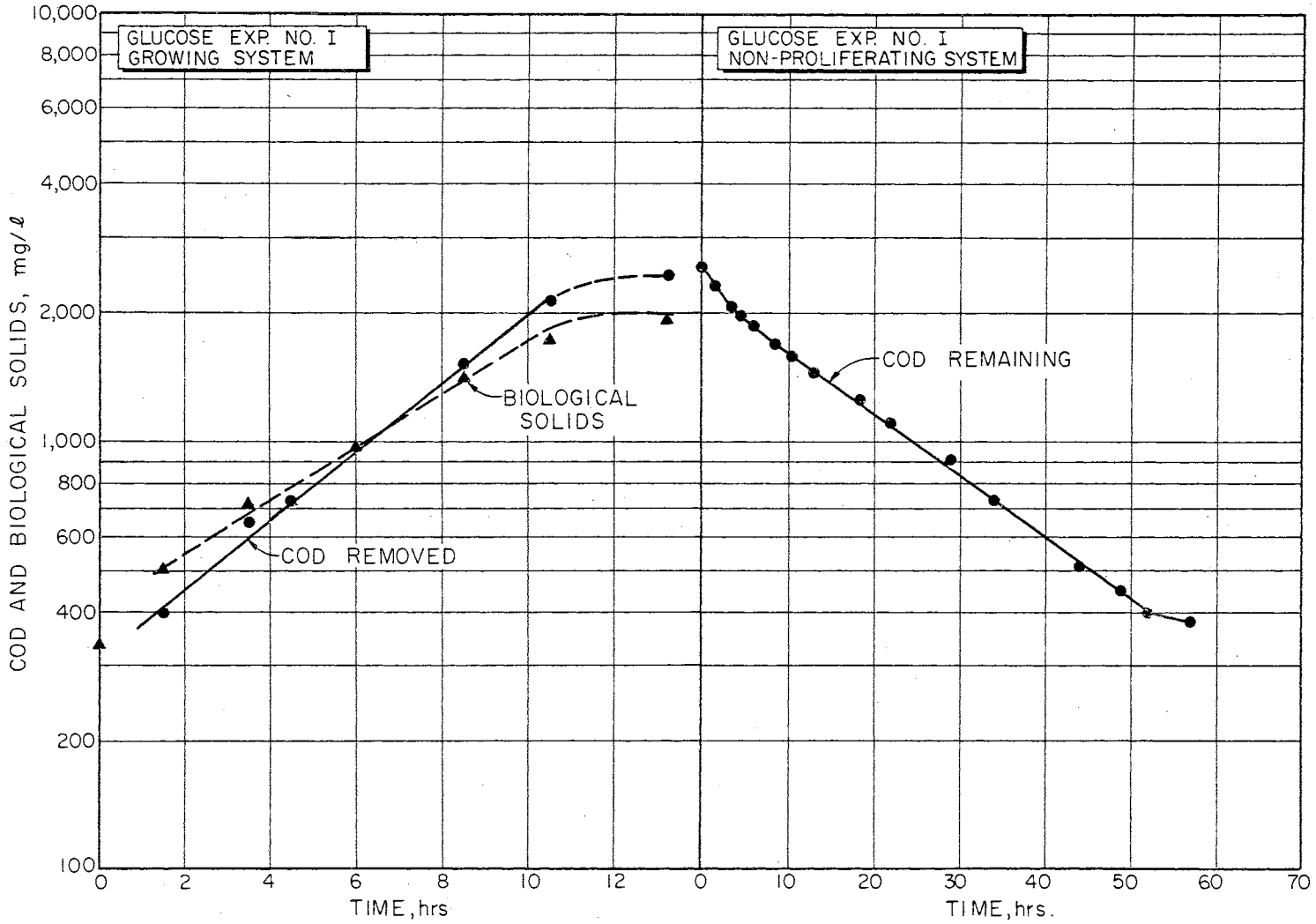


Figure 9 - Semi-log plot of COD removal and solids growth in systems shown in Figure 8.

TABLE VI

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF GLUCOSE (Exp. No.I)

Time Elapsed Hrs:Min	Intermediates and/or Endproducts - COD, mg/l							
	Growing System				Nonproliferating System			
	Total	Acetic Acid	Pyruvic Acid	Others	Total	Acetic Acid	Pyruvic Acid	Others
00.00	-	-	-	-	-	-	-	-
00.20	-	91	3	-	-	165	-	-
01.30	100	91	3	7	180	147	27	6
03.30	-	24	8	-	700	135	50	515
04.30	30	14	-	17	-	88	-	-
06.00	-	11	trace	-	-	75	-	-
08.30	220	13	12	195	500	71	53	376
10.30	326	6	12	308	-	75	-	-
*13.15	28	19	trace	9	495	-	104	-
18.30	38	15	"	23	785	92	98	595
22.00	76	nil	nil	76	730	100	114	516
29.00	46	"	"	46	445	104	66	275
34.00	-	-	-	-	539	75	55	409
44.15	-	-	-	-	-	62	54	-
48.45	-	-	-	-	312	-	43	-
**51.50	-	-	-	-	400	45	34	321
56.40	-	-	-	-	380	37	22	321

*Glucose eliminated from medium in the growing system.

**Glucose eliminated from medium in the nonproliferating system.

growing system, they persisted in the medium of the nonproliferating system even after prolonged aeration.

Carbohydrate synthesis was predominant during the solids accumulation phase of the growing system. It peaked at the time of maximum solids synthesis, and accounted for 48 per cent of the net solids increase. More than 40 per cent of the carbohydrate accumulated during the substrate removal phase was utilized by the cells during the first sixteen hours of endogenous phase. Protein synthesis reached the maximum level in the early endogenous phase. There was no increase in the lipid content of the cells during growth on glucose.

Carbohydrate accounted for 81 per cent of the solids accumulated under nonproliferating conditions. The total carbohydrate accumulation amounted to as much as three times the initial dry weight of the cells. An insignificant amount of protein synthesis (0.7 per cent of the net solids increase) was noted in the system. No lipid accumulation was observed. The cells contained 73 per cent carbohydrate and six per cent protein at the end of 52 hours.

Maximum ammonia nitrogen removal from the medium of the growth system coincided with the time of peak in protein synthesis. One hundred fifty mg/l of ammonia nitrogen had been utilized for growth. No organic nitrogen was detected in the external medium of the system. Analysis of the membrane filtrate of the nonproliferating system for ammonia or oganic nitrogen indicated that neither form of nitrogen was present in the system at any time during the course of the experiment.

b. Experiment II. Metabolism of Glucose by Growing and Nonproliferating Cells at an Intermediate Ratio of Initial COD:Biological Solids

Figure 10 shows the response when the initial COD:biological solids

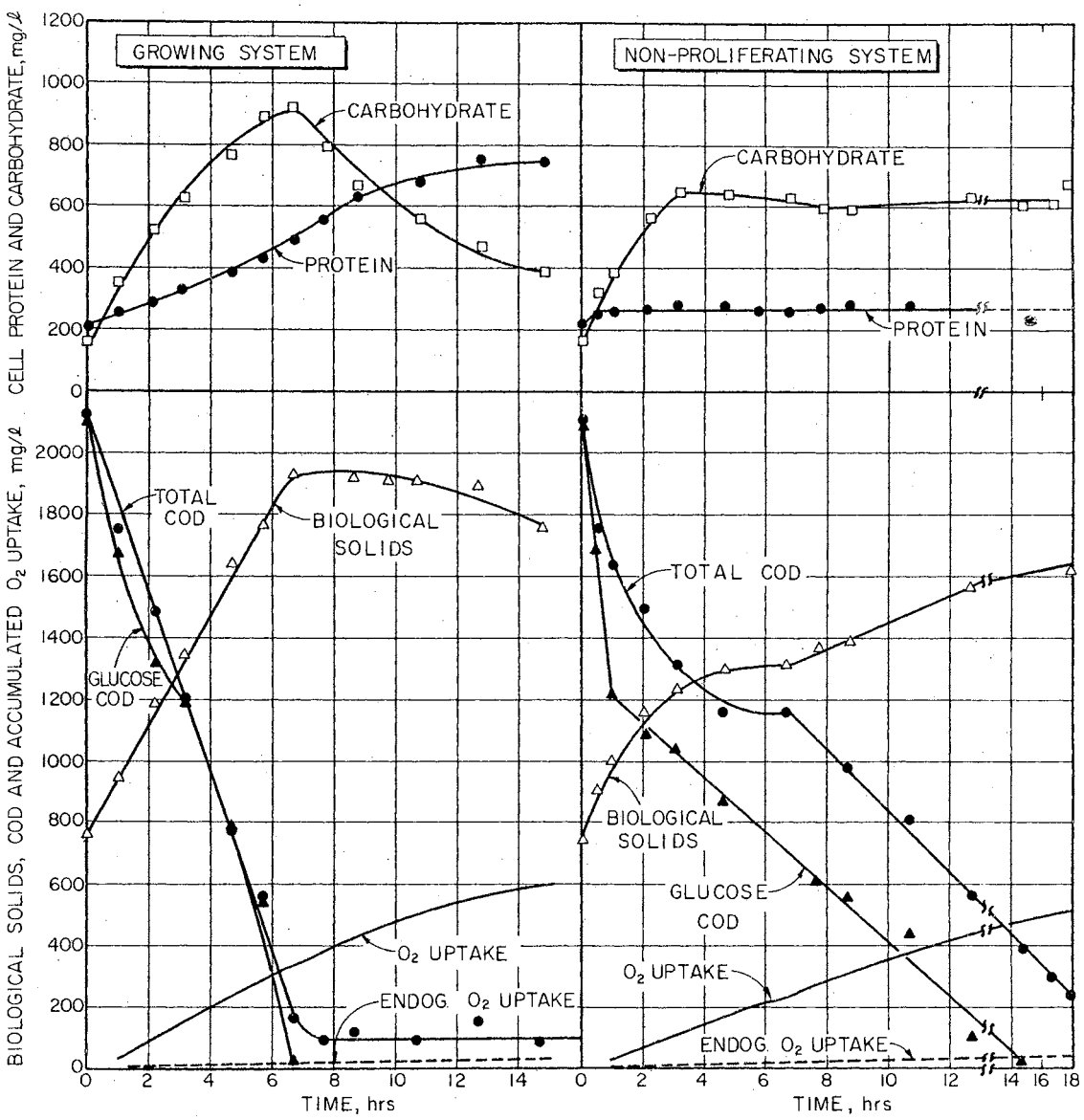


Figure 10 - Response of growing and nonproliferating systems during metabolism of glucose at intermediate ratio of initial COD:biological solids (Experiment II).

ratio of 2.8 was employed. COD removal and solids growth followed linear or zero order kinetics throughout the solids accumulation phase in the growth system. However, under nonproliferating conditions, COD removal and solids growth were composed of two distinct phases of first order kinetics. Figure 11 shows the semi-log plot of COD remaining versus time in this system. There was a lag of about three hours between the two phases during which no COD removal or solids growth took place. This lag phase may represent the acclimation of the cells to the metabolic intermediates released during the earlier phase of glucose removal or a sequential removal of the substrate by different populations.

Very small amounts of metabolic intermediates were released by the growing cells. A maximum of 170 mg/l COD (eight per cent of the initial COD) was present as non-glucose COD during the early growth phase. The residual COD at tne end of the experiment (15 hours) amounted to four per cent of the initial COD. Under nonproliferating conditions as much as 470 mg/l COD (22 per cent of the initial) was present in the medium as metabolic intermediates. The residual COD at the time of termination of the experiment amounted to 11 per cent of the initial value.

Accumulation of carbohydrates was the major synthetic activity observed in both systems. Carbohydrate amounted to 65 per cent of the solids synthesized in the growing system, and 59 per cent in the nonproliferating system. It is interesting to note that carbohydrate accounted for the total amount of solids synthesized during the initial phase of substrate removal in the latter system. The secondary substrate removal phase did not involve the synthesis of carbohydrate.

Carbohydrate served as the endogenous carbon source for protein

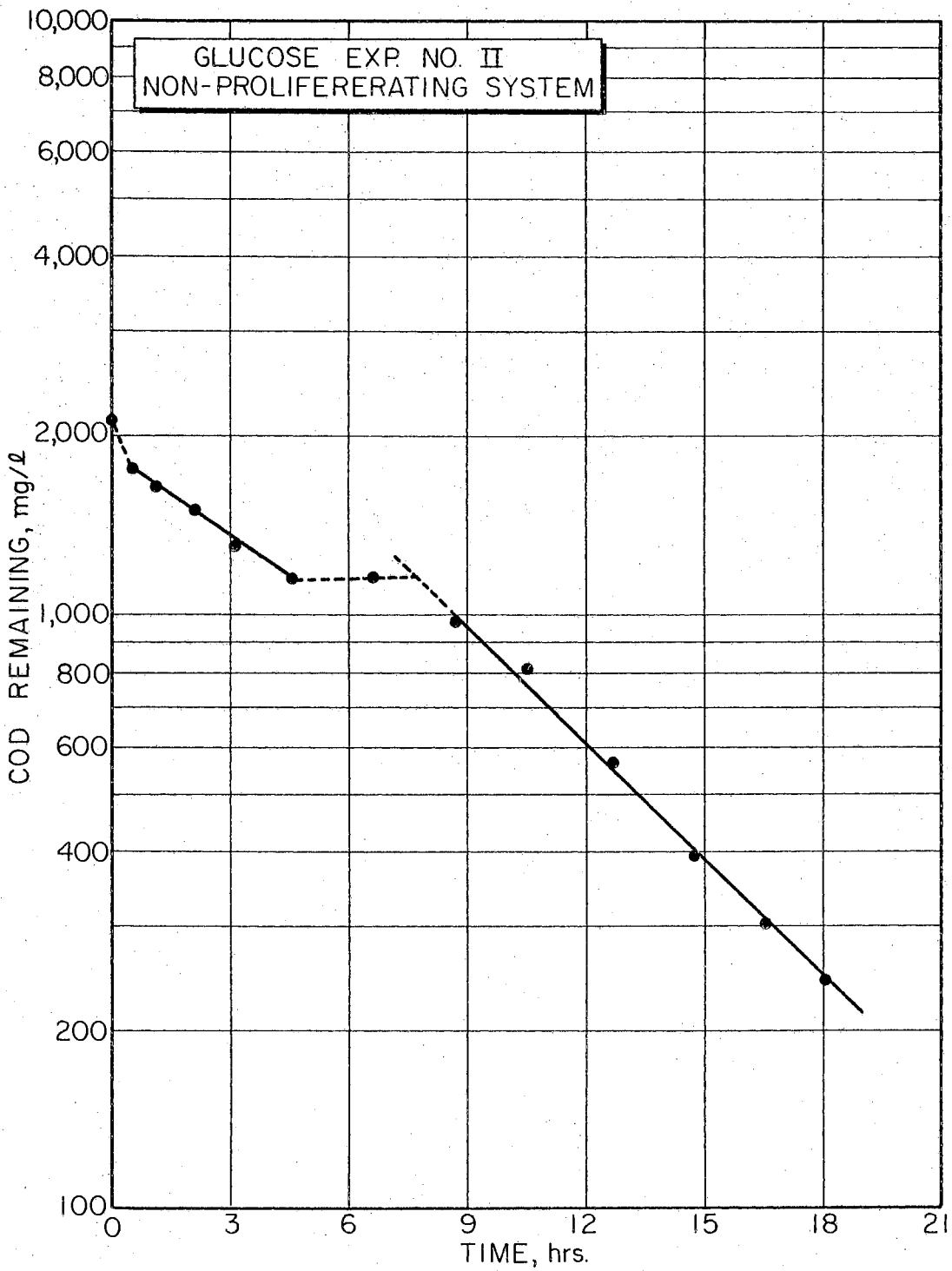


Figure 11 - Semi-log plot of COD remaining in nonproliferating system shown in Figure 10.

synthesis in the growth system. Seventy per cent of the carbohydrate synthesized during the substrate removal phase was degraded during the first eight hours of the endogenous phase in this system. A small initial increase in protein was noted in the nonproliferating system.

The accumulated O_2 uptake curve showed two linear uptake phases separated by a plateau. Sixteen per cent of the theoretical O_2 demand was exerted at the time of substrate removal (93 per cent COD removal) in the growing system. Twenty-four per cent of the theoretical O_2 demand was exerted in the nonproliferating system up to the end of the experiment (89 per cent COD removal). Table VII shows the materials balance calculations for the two systems. Substrate recoveries were generally in the range of 80 to 90 per cent.

c. Experiment III. Metabolism of Glucose by Growing and Nonproliferating Cells at Low Initial COD:Biological Solids Ratio

Figure 12 shows the response of the systems at an initial COD: solids ratio of 1.3. There was practically no difference in the COD removal and solids growth patterns between two systems. Both of these parameters showed linear kinetics during the substrate removal phase of either system. The cell yield was slightly higher under nonproliferating conditions.

Very small amounts of intermediates were released in the growing system. A maximum of eight per cent of the initial COD was present as non-glucose COD in the growing system, and 14 per cent in the nonproliferating system. A major difference between the two systems is the type and amount of cellular constituents synthesized during the metabolism of glucose. Carbohydrate synthesis amounted to 69 per cent of the solids accumulated under growing conditions, and 87 per cent under

TABLE VII

MATERIALS BALANCE FOR METABOLISM OF GLUCOSE (Exp. No.II)

Time Elapsed Hrs:Min	COD mg/l	Accumulated Decrease in COD, mg/l	COD* Calculated as Substrate mg/l	Solids mg/l	Accumulated Increase in Solids mg/l	Accumulated O_2 Uptake mg/l	Substrate Respired* mg/l	Percent Recovery
				Growing System				
00.00	2120	-	-	758	-	-	-	-
00.30	1840	280	260	-	-	-	-	-
01.00	1750	370	344	943	185	33	31	75
02.10	1490	630	586	1180	422	100	94	88
03.10	1200	920	855	1344	586	150	141	85
04.40	770	1350	1260	1640	882	240	225	88
05.40	560	1560	1450	1766	1008	290	272	85
06.40	160	1960	1820	1934	1176	335	314	77
07.40	90	2030	1890	1918	1160	370	347	80
				Nonproliferating System				
00.00	2110	-	-	746	-	-	-	-
00.30	1760	350	328	904	158	-		
01.00	1640	470	440	1008	262	30	28	66
02.10	1500	610	570	1155	409	70	66	83
03.10	1320	790	740	1229	483	120	112	81
04.40	1160	950	890	1295	549	170	159	80
05.40	-	-	-	-	-	-	-	-
06.40	1170	940	880	1318	572	230	216	90
07.40	-	-	-	1363	617	270	253	-
08.40	980	1130	1060	1384	638	310	290	88
10.40	810	1300	1220	-	-	370	347	-
12.40	560	1550	1450	1567	821	430	402	84
14.40	390	1720	1610	-	-	470	440	-

*Expressed as equivalent glucose.

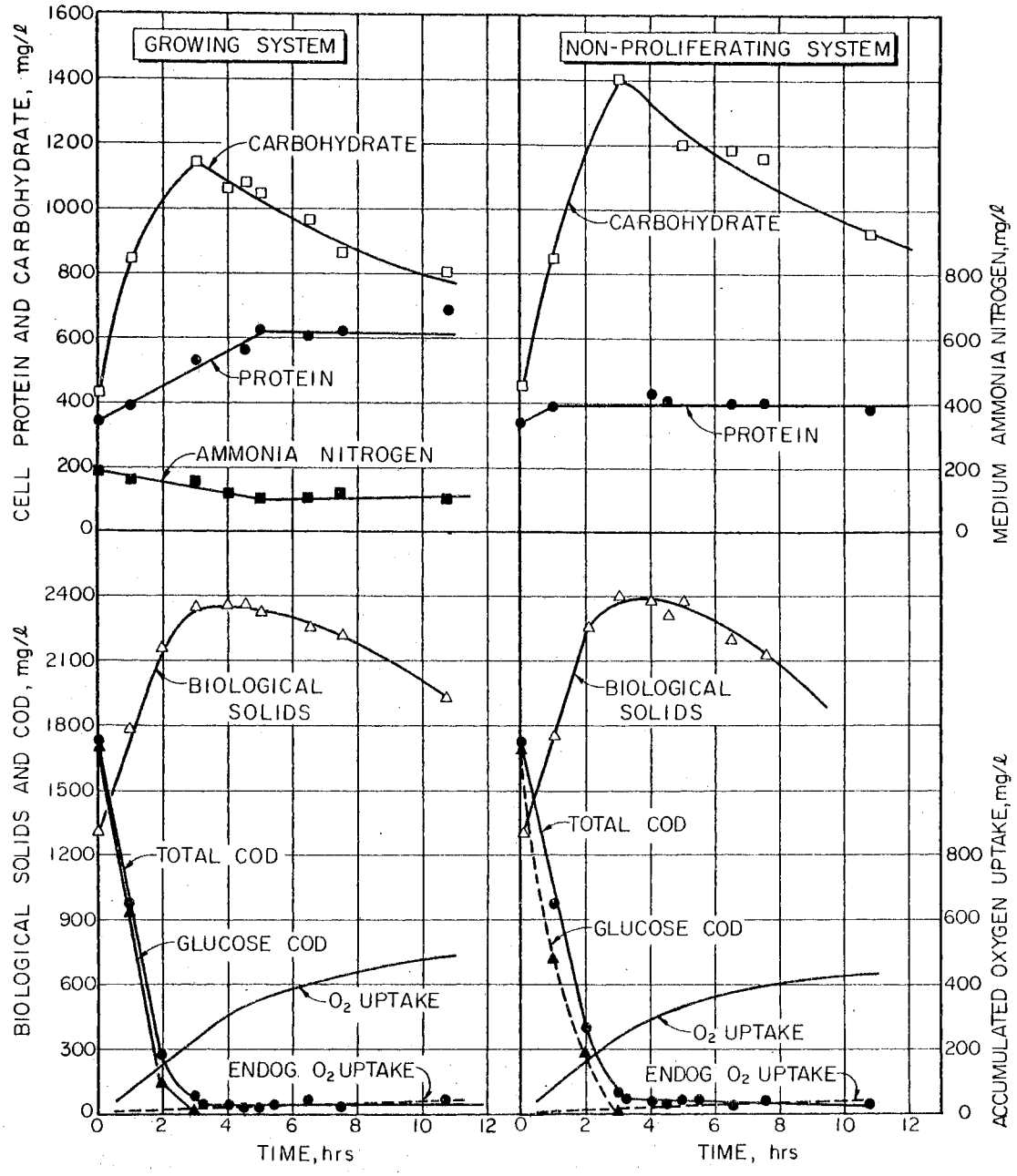


Figure 12 - Response of growing and nonproliferating systems during metabolism of glucose at low initial COD:biological solids ratio (Experiment III).

nonproliferating conditions. Protein content reached a maximum in the early endogenous phase of the growth system. Endogenous utilization of cellular carbohydrate was comparable in both systems. About 50 per cent of the carbohydrate accumulated in the substrate removal phase was degraded in both cases. At the end of the experiment (10 hours and 45 minutes) the carbohydrate content of the cells in both systems was 42 per cent. Whereas the protein content of the cells in the growth system was 35 per cent, the nonproliferating cells contained only 17 per cent protein.

Ninety mg/l of ammonia nitrogen was taken up from the medium by the growing cells. No organic nitrogen-containing materials could be detected in the medium of either system. Ammonia nitrogen was absent in the nonproliferating system throughout the course of the experiment. Substrate respiration was the same in both systems. The theoretical O_2 demand exerted at the time of substrate removal (95 per cent COD removal) was 13.4 per cent in the growing system, whereas in the nonproliferating system 13.8 per cent of theoretical O_2 demand was exerted under similar conditions.

Table VIII shows the materials balance calculations for these systems. Substrate recovery ranged from 72 to 87 per cent.

2. Metabolism of Sucrose

a. Experiment I. Preliminary Study

The results obtained in a preliminary study are presented in Figure 13. There was a rapid initial uptake of COD in both systems. The major point of interest in this study is the appearance of large amounts of glucose (as measured by the Glucostat method) in the external medium of both growing and nonproliferating systems,

TABLE VIII

MATERIALS BALANCE FOR METABOLISM OF GLUCOSE (Exp. No.III)

Time Elapsed Hrs:Min	COD mg/l	Accumulated Decrease in COD, mg/l	COD* Calculated as Substrate mg/l	Solids mg/l	Accumulated Increase in Solids mg/l	Accumulated O_2 Uptake mg/l	Substrate Respired* mg/l	Percent Recovery
				Growing System				
00.00	1720	-	-	1300	-	-	-	-
01.00	980	740	693	1780	480	76	71	80
02.00	279	1441	1350	2160	860	157	147	74
03.00	87	1633	1530	2340	1040	231	216	82
03.15	48	1672	1570	-	-	-	-	-
04.00	45	1675	1570	2365	1065	306	287	86
04.30	24	1696	1590	2350	1050	333	312	86
05.00	32	1698	1590	2310	1010	355	333	85
05.25	45	1675	1580	-	-	370	343	-
06.30	68	1652	1550	2250	950	402	376	85
07.30	26	1694	1590	2222	922	430	403	83
10.45	70	1650	1550	1925	625	490	460	82
				Nonproliferating System				
00.00	1730	-	-	1310	-	-	-	-
01.00	970	760	712	1750	440	75	70	72
02.00	394	1336	1250	2250	940	151	141	87
03.00	96	1634	1530	2400	1090	220	206	80
03.15	72	1658	1550	-	-	-	-	-
04.00	57	1673	1565	2375	1065	275	257	84
04.30	36	1694	1585	2300	990	296	277	80
05.00	58	1672	1570	2380	1070	317	296	87
05.25	58	1672	1570	-	-	345	320	-
06.30	35	1695	1585	2190	880	360	337	77
07.30	51	1679	1572	2125	815	390	366	75
1045	43	1687	1580	2190	880	439	412	82

*Expressed as equivalent glucose

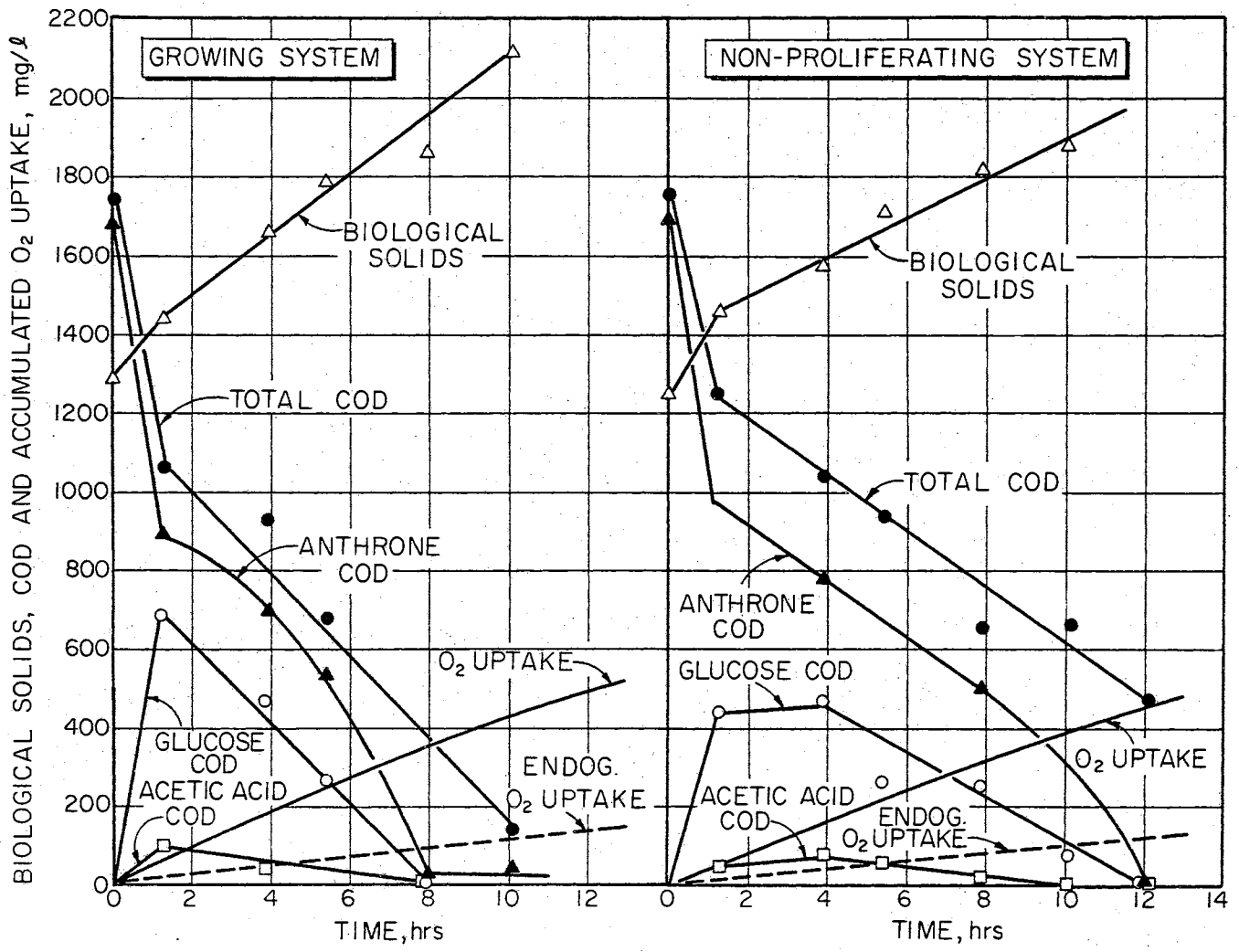


Figure 13 - Metabolism of sucrose under growing and nonproliferating conditions (Experiment I).

indicating rapid extracellular hydrolysis of sucrose by the cells, irrespective of the presence or absence of exogenous nitrogen. The anthrone COD indicated in the figures represents the total carbohydrates in the medium, and in view of the Glucostat results may be regarded as only a gross oversimplification of the actual metabolic fate of sucrose.

Acetic acid accumulated in both systems during the early phase of sucrose metabolism, and was metabolized by the cells concurrently with the other substrates. In order to determine the fate of sucrose, similar experiments were carried out after standardizing the analytical methods for the determination of the individual sugars. The cells employed in all experiments were obtained from the same stock reactor.

b. Experiment II

Figure 14 shows the results of one such experiment in which the initial COD:biological solids ratio was 2.4. As observed in the preliminary experiment, there was a rapid decrease in total COD in both systems during the first hour. Subsequent removal of COD was linear in the growing system and followed decreasing first order rate kinetics in the nonproliferating system (Figure 16). The rapid initial decrease in COD was due to the sudden uptake of sucrose by the cells, and this process did not depend upon the presence of exogenous nitrogen. It is seen that large amounts of glucose appeared in the medium of both systems. Only small amounts of fructose were detected in the growing system; however, under nonproliferating conditions, glucose and fructose were released into the medium in approximately equal amounts, although appearance of glucose preceded fructose. Both were removed concurrently in this system. Table IX shows the amounts of various metabolic intermediates present in the systems at various times. More

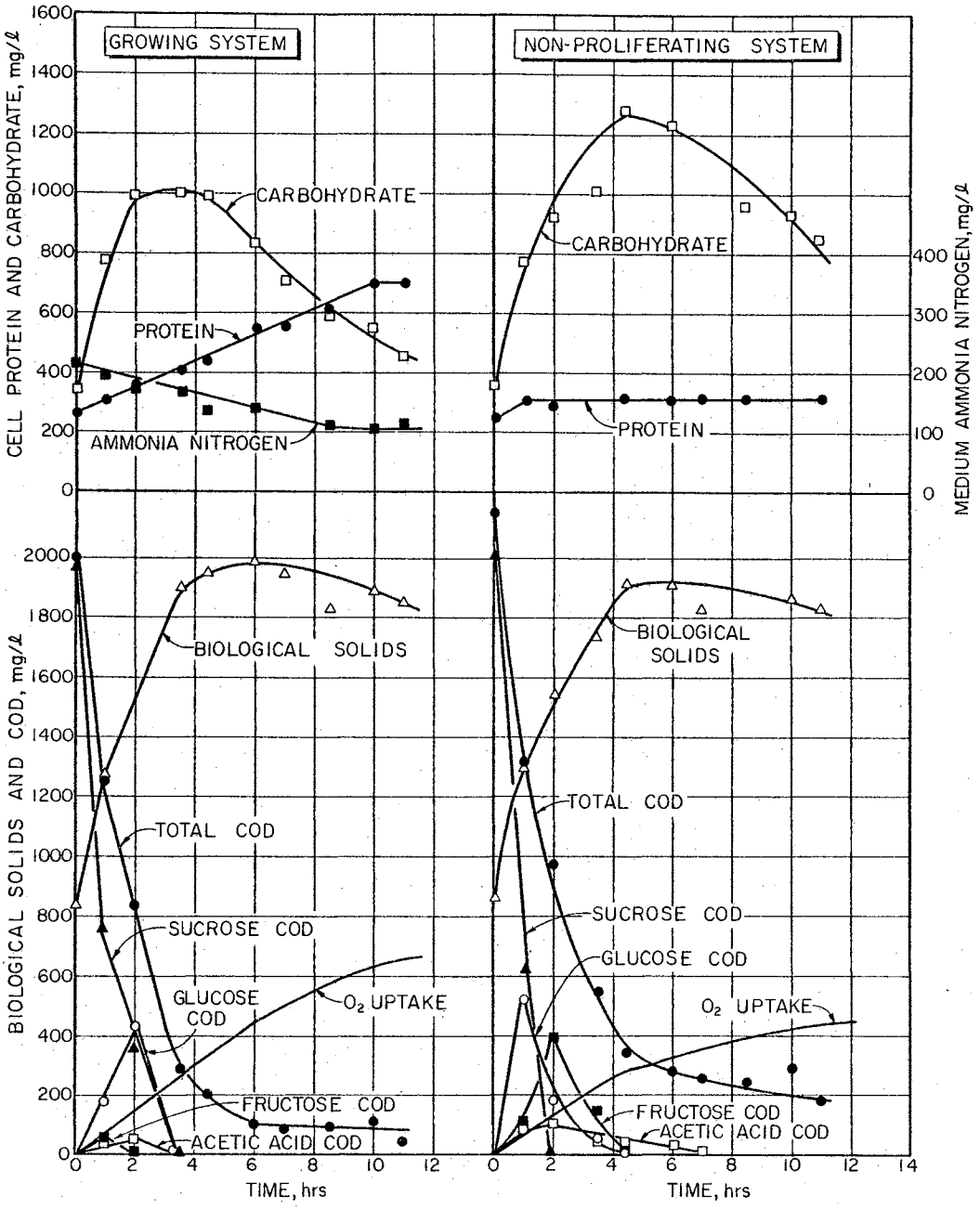


Figure 14 - Metabolism of sucrose under growing and nonproliferating conditions (Experiment II).

TABLE IX

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF SUCROSE (Exp. No. II)

Time Elapsed Hrs:Min	Intermediates and/or Endproducts COD, mg/l				
	Total	Free Glucose	Free Fructose	Acetic Acid	Others
			Growing System		
00.00	-	-	-	-	-
01.00	510	181	42	40	247
02.00	486	435	nil	53	nil
03.30	295	nil	"	nil	284
04.25	-	"	"	"	-
06.00	103	"	"	"	103
07.00	90	"	"	"	90
08.30	90	"	"	"	90
10.00	112	"	"	"	112
11.00	38	"	"	"	38
			Nonproliferating System		
00.00	-	-	-	-	-
01.00	705	520	106	80	nil
02.00	975	170	405	107	293
03.30	550	53	148	43	306
04.25	346	nil	nil	24	322
06.00	280	"	"	18	262
07.00	252	"	"	nil	252
08.30	240	"	"	"	240
10.00	292	"	"	"	292
11.00	180	"	"	"	180

intermediates (carbohydrate as well as non-carbohydrate) were released in the nonproliferating system. Acetic acid was released in both systems during the rapid breakdown of sucrose in the first two hours. About twice as much acetic acid was released in the nonproliferating system as in the growing system. The sugars and acetic acid were removed subsequently under both conditions; however, more endproducts were present in the nonproliferating system at the termination of the experiment.

Oxygen uptake proceeded linearly in both systems, and the O_2 uptake curves exhibited a decreased slope after the time of maximum solids accumulation in both systems. At this point, 22.2 per cent of initial COD (theoretical O_2 demand) was oxidized in the growing system (95 per cent COD removal), and 15.1 per cent in the nonproliferating system (87 per cent COD removal).

Table X shows the materials balance calculations for the systems. Substrate recoveries ranged from 73 to 93 per cent. More carbohydrate accumulation occurred under nonproliferating conditions. As much as 83 per cent of the solids synthesized in the absence of nitrogen was carbohydrate. It can be seen that most of the protein synthesis in the growing system occurred in the early endogenous phase. Carbohydrates were degraded rapidly while the protein synthesis was in progress. About 80 per cent of the accumulated carbohydrate was utilized during eight hours of endogenous respiration. The relative constant cell dry weight during the early endogenous phase indicates that protein synthesis occurred at the expense of the stored carbohydrate. Only 46 per cent of the stored carbohydrate was oxidized during the first seven hours of endogenous metabolism in the nonproliferating system. At the

TABLE X

MATERIALS BALANCE FOR METABOLISM OF SUCROSE (Exp. No. II)

Time Elapsed Hrs:Min	COD mg/l	Accumulated Decrease in COD, mg/l	COD* Calculated as Substrate mg/l	Solids mg/l	Accumulated Increase in Solids mg/l	Accumulated O_2 Uptake mg/l	Substrate Respired* mg/l	Percent Recovery
				Growing System				
00.00	2000	-	-	835	-	-	-	-
01.00	1270	730	685	1290	455	70	66	76
02.00	840	1160	1090	-	-	150	141	-
03.30	295	1705	1600	1900	1065	260	244	82
04.25	-	-	-	1960	1125	336	316	-
06.00	103	1897	1780	1980	1145	445	417	88
07.00	90	1910	1790	1940	1105	500	470	88
08.30	90	1910	1790	1830	995	570	535	86
10.00	112	1888	1765	1880	1045	630	591	93
11.00	38	1962	1840	1850	1015	655	615	99
				Nonproliferating System				
00.00	2150	-	-	855	-	-	-	-
01.00	1310	840	787	1310	455	65	61	66
02.00	975	1175	1100	1540	685	130	122	73
03.30	550	1600	1510	1740	885	230	216	73
04.25	346	1804	1700	1920	1065	277	260	78
06.00	280	1870	1760	1900	1045	325	305	77
07.00	252	1898	1780	1820	965	355	333	73
08.30	240	1910	1790	1800	945	395	370	74
10.00	292	1858	1750	1870	1015	422	397	81
11.00	180	1970	1850	1830	975	437	410	75

*Expressed as equivalent sucrose.

end of the experiment (eleven hours) the nonproliferating cells contained twice as much carbohydrate and half as much protein as the cells in the growing system.

Ammonia nitrogen utilization reached a maximum at the time of the peak in protein synthesis. No ammonia nitrogen was detected in the medium of the nonproliferating system. Organic nitrogen compounds were absent in the medium of both systems.

c. Experiment III

Figure 15 shows the results obtained in another experiment on sucrose with an initial COD:biological solids ratio of about 2.3. A rapid initial COD uptake, followed by a linear removal, was evident in the growing system. However, under nonproliferating conditions, COD removal followed decreasing first order kinetics after an initial linear uptake of COD (Figure 16). Cell yield was lower in the nonproliferating system. Maximum solids yield in the nonproliferating system was obtained when only 63 per cent of the initial COD had been removed from the medium (seven hours). Further decrease in COD did not contribute to an increase in solids. The fate of sucrose in these systems was similar to that in the previous experiment, with one difference; no free sucrose COD was detected in the external medium of either system in this experiment at the end of the first 30 minutes of aeration. It was completely hydrolyzed and was present as its constituent monosaccharides, glucose, and fructose. Although glucose accumulated in larger amounts than fructose in both systems, it was removed at a much faster rate than was fructose. Fructose removal was very much retarded under nonproliferating conditions. A considerable amount of fructose remained in the medium at the termination of the experiment (22 hours).

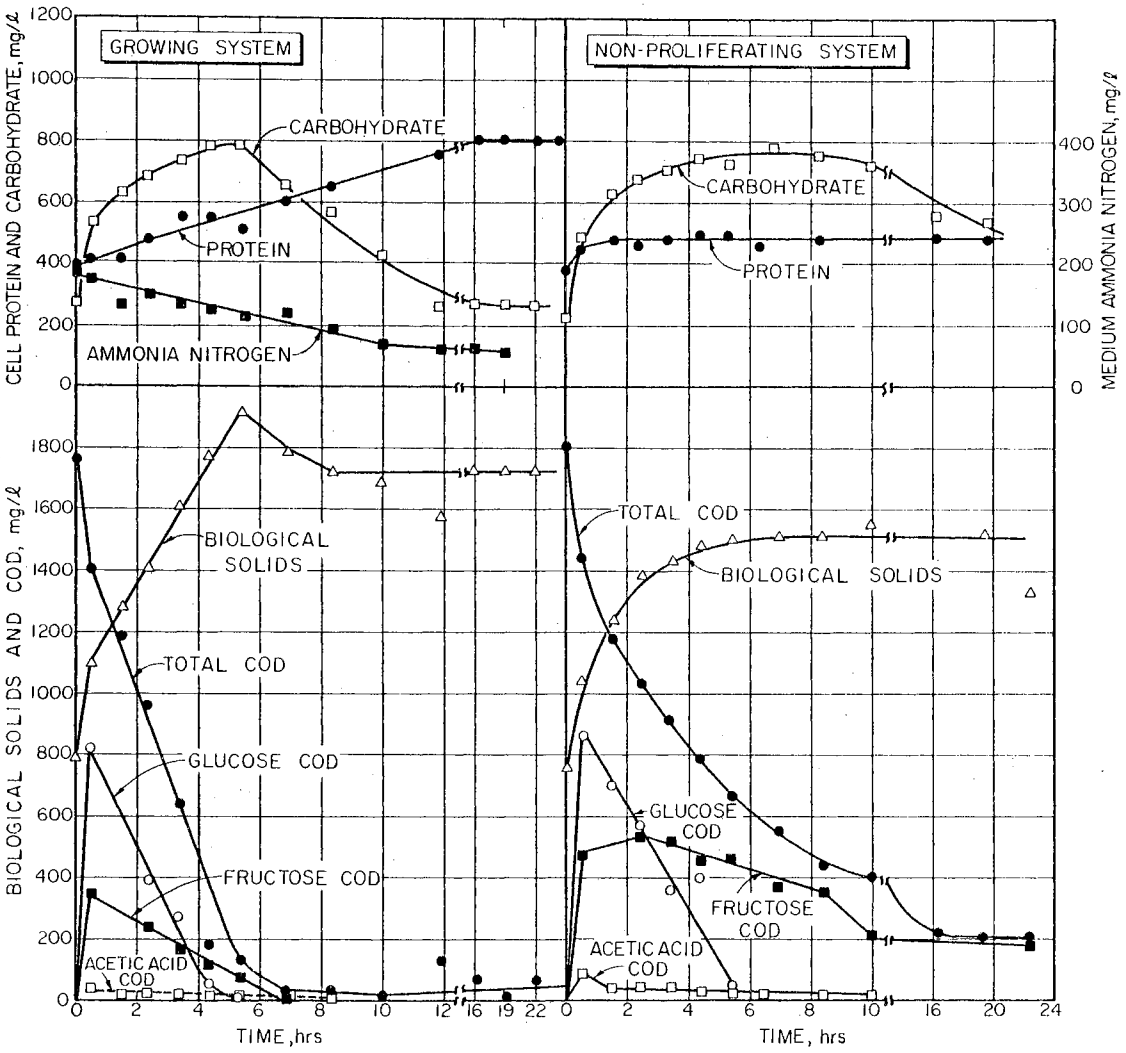


Figure 15 - Metabolism of sucrose under growing and nonproliferating conditions (Experiment III).

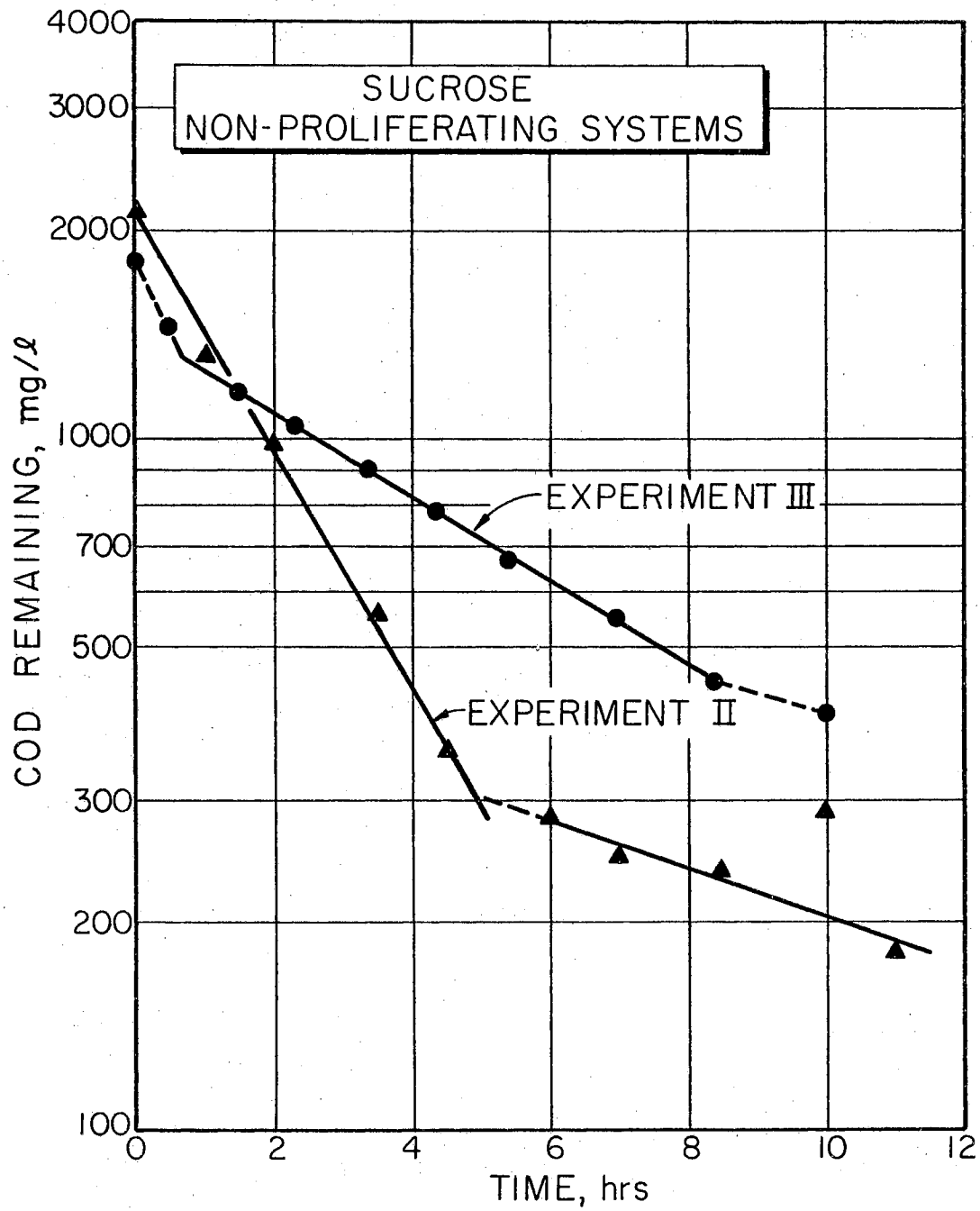


Figure 16 - Semi-log plot of COD remaining in nonproliferating systems shown in Figures 14 and 15.

Table XI shows the amounts of various intermediates of sucrose metabolism under growing and nonproliferating conditions.

Acetic acid accumulated during the first 30 minutes of sucrose metabolism under both conditions. Slightly higher amounts were released by the nonproliferating cells.

Synthesis of carbohydrate and protein under growth conditions was similar to the earlier experiment. One hundred twenty one mg/l of ammonia nitrogen were utilized by the cells. It is interesting to note the behavior of the nonproliferating system with regard to the synthesis of cellular carbohydrate. Sixty-three per cent of the solids accumulation in the system was due to carbohydrate synthesis which apparently reached the maximum limit before the exhaustion of the exogenous substrate. About 30 per cent of the initial COD was still present in the medium at the time of the peak in carbohydrate content. The biological solids level also reached the maximum limit at approximately the same time. Further COD removal did not contribute to an increase in solids in the system. It is also seen that the system could not metabolize the fructose remaining in the medium after the termination of carbohydrate synthesis. Total degradation of cellular carbohydrate accumulated during the substrate removal phase was observed during the first seven hours of endogenous metabolism in the growing system. Cell carbohydrate remained stationary in the nonproliferating system until nearly all of the COD had been removed, and subsequently about 40 per cent of the amount accumulated earlier was oxidized during the limited endogenous phase observed in the experiment.

3. Metabolism of Lactose

Figure 17 shows the metabolic response of the lactose system under

TABLE XI

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF SUCROSE (Exp. No. III)

Time Elapsed Hrs:Min	Intermediates and/or Endproducts COD, mg/l				
	Total	Free Glucose	Free Fructose	Acetic Acid	Others
			Growing System		
00.00	-	-	-	-	-
00.30	1282	820	345	38	82
01.30	1185	1060	106	24	5
02.20	965	388	237	21	319
03.20	645	270	165	22	188
04.20	185	57	116	12	nil
05.20	130	nil	87	11	32
06.50	35	"	nil	13	22
08.20	35	"	"	nil	35
10.00	15	"	"	"	15
11.50	130	"	"	"	130
			Nonproliferating System		
00.00	-	-	-	-	-
00.30	1435	860	470	72	33
01.30	1170	700	-	34	-
02.20	1045	570	540	42	nil
03.20	910	360	520	36	"
04.20	785	-	450	27	-
05.20	665	42	470	23	130
06.50	555	nil	370	12	173
08.20	445	"	350	16	79
10.00	405	"	210	9	186
11.50	-	"	170	12	-
16.20	225	"	-	nil	-
19.20	215	"	-	"	-
22.20	208	"	170	"	38

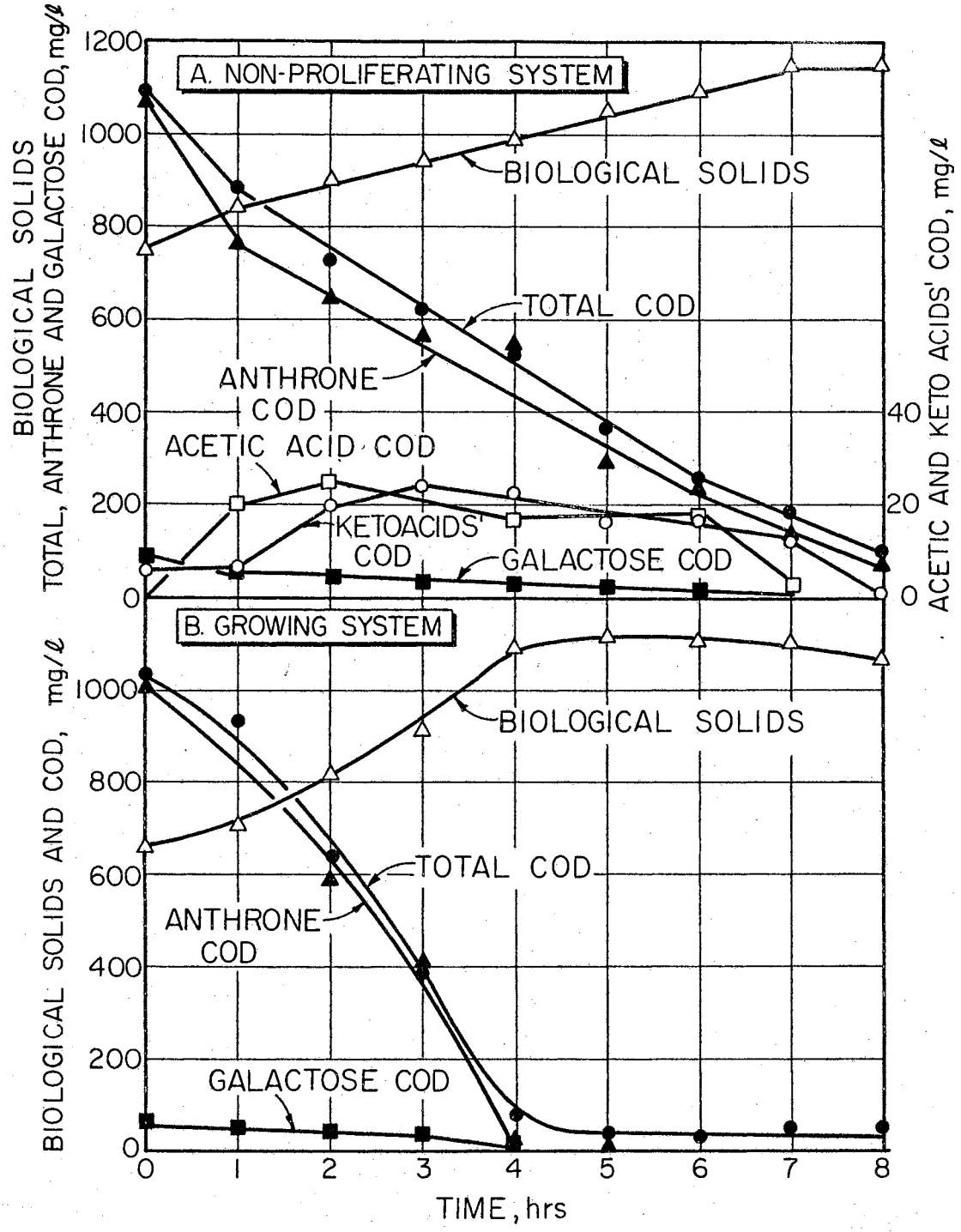


Figure 17 - Metabolism of lactose under growing and nonproliferating conditions.

growing and nonproliferating conditions, at initial COD:solids ratios of 1.60 and 1.45, respectively. Data for the total COD and biological solids are from the unpublished results of Painter and Gaudy (144). Anthrone COD represents the total carbohydrate COD in the systems. Lactose was used as the standard for the anthrone test. Since no glucose and only very small amounts of galactose (Table XII) were present in the systems, the anthrone COD can be taken as the measure of lactose COD in the systems.

The lactose systems provide an interesting contrast to the sucrose systems discussed earlier. It is seen from Table XII that lactose is not hydrolyzed extracellularly into the monosaccharide units (or in any event, the constituent monosaccharides did not accumulate). Small amounts of galactose were detected in the systems even at the beginning of the experiment. Probably this represents the presence of galactose as an impurity in the lactose used in the study.

The growing cells produced no detectible amounts of metabolic intermediates. Most of the intermediates released under nonproliferating conditions were accounted for as acetic and keto acids. These were subsequently metabolized by the cells.

4. Metabolism of Volatile Fatty Acids

As reported in the earlier sections of this report, acetic acid is a commonly occurring intermediate during the metabolism of glucose, sucrose, and lactose, by growing as well as nonproliferating cells. Acetic and propionic acids were also released during the metabolism of polyhydric alcohols like sorbitol and glycerol (to be discussed later in this report).

Non-ionized or free acetic acid as well as other lower free fatty

TABLE XII

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF LACTOSE

Time Elapsed Hrs	Metabolic Intermediates and/or Endproducts COD, mg/l				
	Total	Free Glucose	Free Galactose	Acetic Acid	Keto Acids
			Growing System		
0	-	nil	61	nil	4
1	-	"	51	"	4
2	44	"	36	"	3
3	40	"	36	"	2
4	46	"	nil	"	nil
5	21	"	"	"	"
6	16	"	"	"	"
7	29	"	"	"	"
8	21	"	"	"	"
			Nonproliferating System		
0	-	nil	80	nil	6
1	123	"	48	21	6
2	82	"	40	25	20
3	56	"	34	nil	24
4	-	"	28	16	23
5	67	"	19	nil	17
6	22	"	19	18	19
7	49	"	10	nil	13
8	29	"	nil	"	2

acids have been reported to kill, or inhibit the growth of, many bacteria (145)(146). However, the ionized form has been found to be less toxic to growth. It seemed, therefore, that even a small decrease in pH of the medium during growth of the cells on carbohydrates and polyhydric alcohols might adversely affect the ecological balance existing among the various species in the mixed cultures employed in the present investigation.

Experiments were therefore initiated to study the metabolism of acetic and propionic acids at different initial pH values obtained by suitably increasing the amounts of 1.0 M phosphate buffer in the growth medium. The effect of initial COD:biological solids ratio was also studied with acetic acid systems at identical initial pH. The metabolism of sodium acetate was also investigated. Table XIII gives the experimental outline for these studies. The cells employed in these studies had been well-acclimated to the respective initial pH and substrate concentrations used in the study (Table XIII). The exceptions are noted in the footnotes of the table.

a. Metabolism of Acetic Acid

i) Experiment I. Metabolism of Acetic Acid Under Growing and Nonproliferating Conditions with Low Initial pH

Figure 18 shows the response of the acetic acid systems with an initial COD:biological solids ratio of 10.2 and a pH of 4.5. The systems contained the same amount of phosphate buffer as was used in the normal growth medium (Table I). Solids growth and COD removal did not show a lag in either system, even though the initial pH was decidedly in the acid range. The pH remained below 5.0 during the metabolism of

TABLE XIII

EXPERIMENTAL OUTLINE FOR STUDIES ON THE METABOLISM OF ACETIC AND PROPIONIC ACIDS

Exp. No.	1.0 M Phosphate Buffer ml/1000 mg Substrate/l	Growing System				Nonproliferating System		
		Initial pH	Initial COD mg/l	Initial Biol. Solids mg/l	Initial COD / Initial Biolog. Solids	Initial COD mg/l	Initial Biol. Solids mg/l	Initial COD / Initial Biolog. Solids
				Acetic Acid Systems				
I	10	4.5	2060	201	10.2	2050	180	11.4
*II	50	6.7	950	96	9.9	925	108	8.6
*III	50	6.7	2040	570	3.6	2040	610	3.3
				Sodium Acetate Systems				
I	10	7.2	2000	425	4.7	2050	440	4.7
II	40	7.1	940	275	3.4	1000	320	3.1
				Propionic Acid Systems				
**I	10	4.5	5050	332	15.2	5150	330	15.6
II	20	5.3	3180	632	5.0	3430	590	5.8

*These two sets of experiments were conducted with sludge obtained from the same stock reactor which was acclimated to 2000 mg/l acetic acid.

**Experiment conducted with sludge acclimated to 2000 mg/l propionic acid.

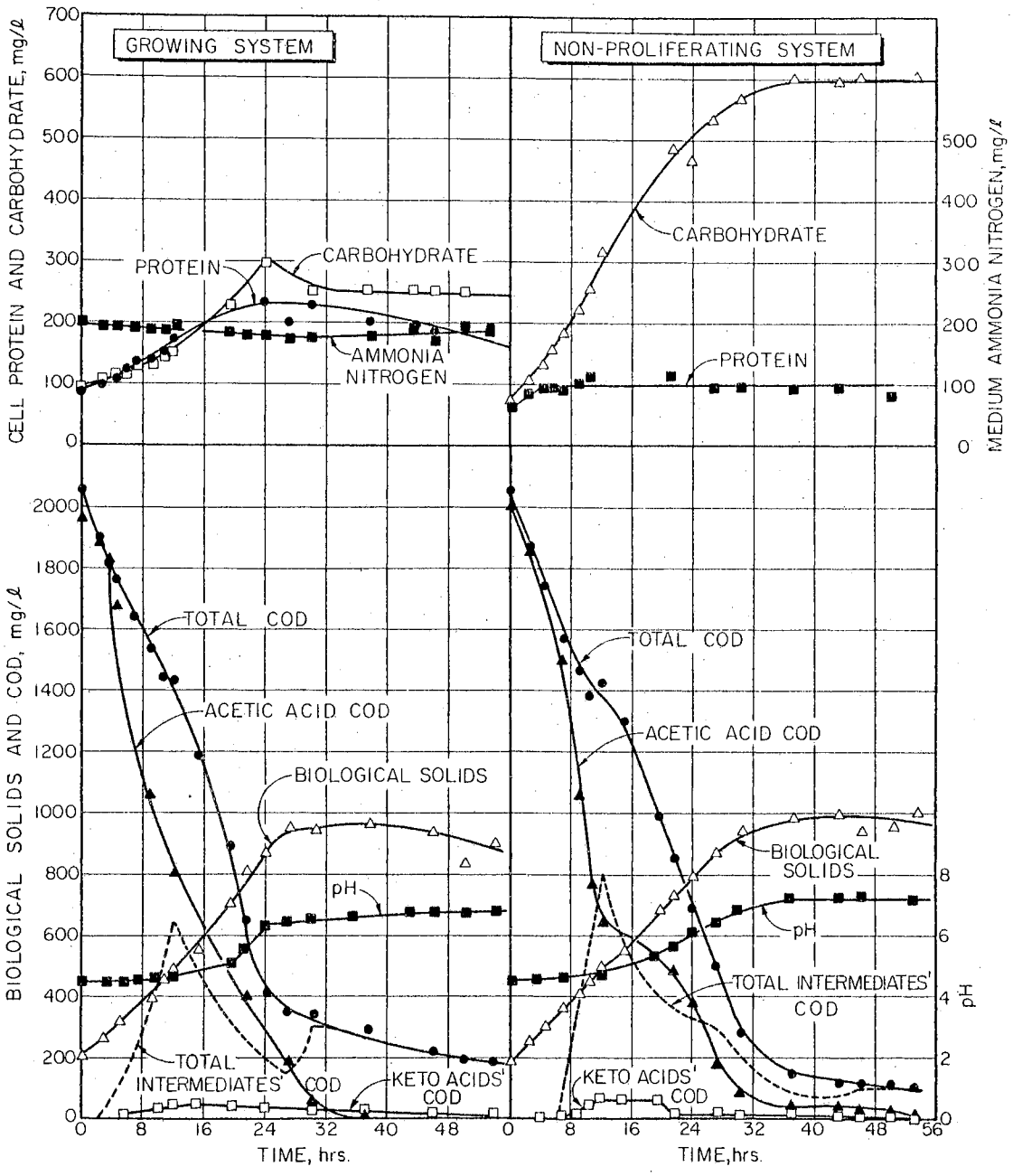


Figure 18 - Metabolism of acetic acid under growing and nonproliferating conditions at low initial pH (Experiment I).

a major part of the acetic acid fed to the system. Growth was exponential in the system supplied with nitrogen. At least two distinct phases of exponential growth were noted (Figure 19). Similarly, in the nonproliferating system, COD removal and solids accumulation exhibited a multiphasic pattern. Solids accumulation was comparable in both systems.

There was a rapid accumulation of metabolic intermediates in both systems during the first twelve hours of acetic acid metabolism. About 30 and 40 per cent of the initial COD was converted into metabolic intermediates during this period in the growing system and nonproliferating system, respectively. The second phase of substrate removal in the growing system (Figure 19) corresponded to the utilization of intermediates accumulated during the initial phase. The phasic removal of COD in the nonproliferating system was also apparently explainable by the removal of intermediates at different rates. Keto acids were released into the medium under both conditions. Small amounts of keto acids persisted in the medium of both systems until the termination of the experiments.

Carbohydrates accounted for only 22 per cent of the solids synthesized in the growth system. The carbohydrate content of the cells was constant (30 to 35 per cent of cell dry weight) throughout the major part of the growth phase. Under nonproliferating conditions, 66 per cent of the accumulated solids could be accounted for as cellular carbohydrate. The carbohydrate content of the cells increased from 40 to 61 per cent during the substrate removal phase. It is interesting to note that very little protein was synthesized in the growing system. Carbohydrate did not serve as endogenous carbon source for continued

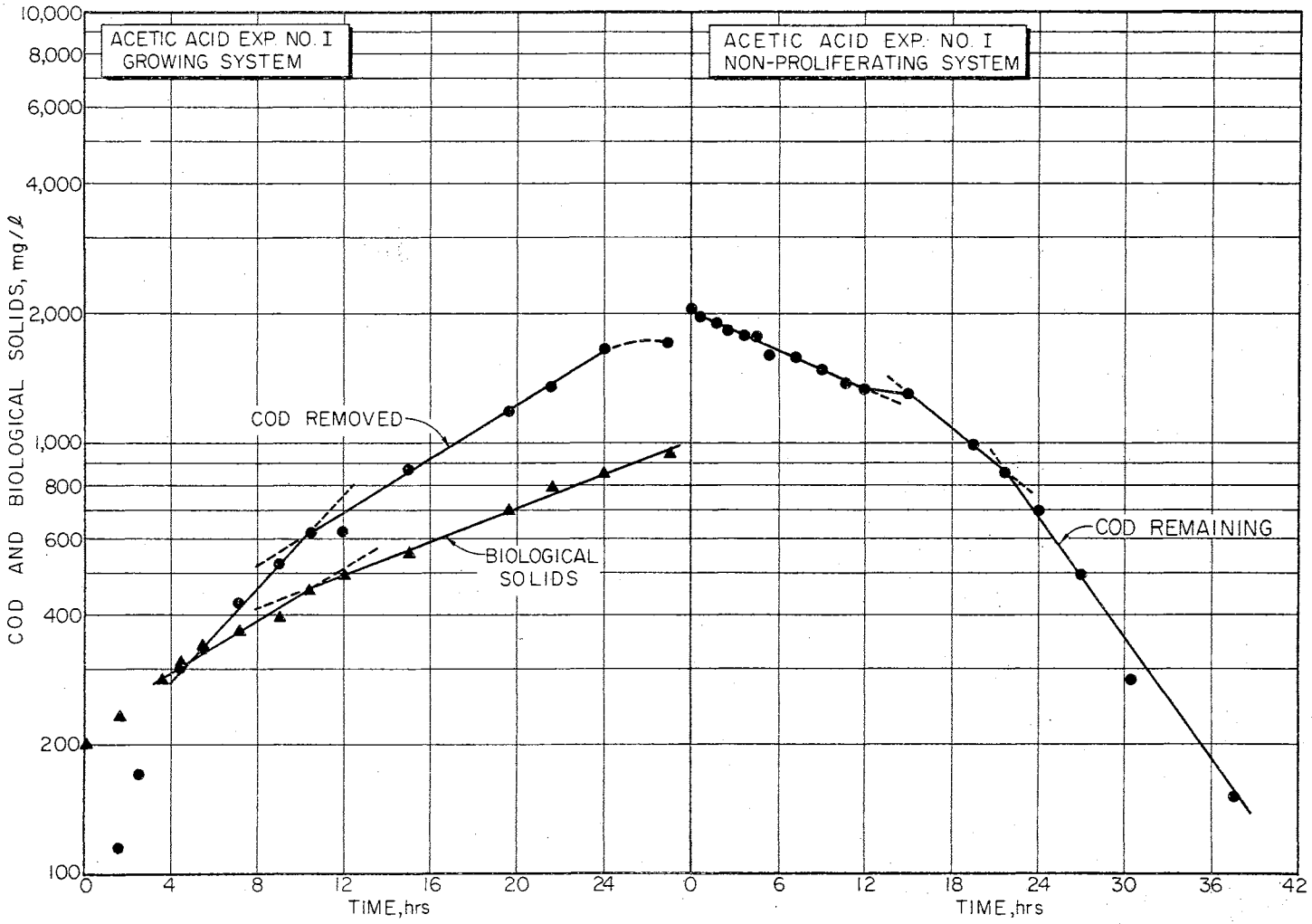


Figure 19 - Semi-log plot of COD removal and solids growth in systems shown in Figure 18.

protein synthesis after exhaustion of the exogenous substrate. Only 28 mg/l of ammonia nitrogen was consumed by the cells during growth. No organic nitrogen-containing materials were released by the growing cells during the course of the experiment. Neither organic nor ammonia nitrogen could be detected in the medium of the nonproliferating system at any time during the experiment.

ii) Experiments II and III. Metabolism of Acetic Acid Under Growing and Nonproliferating Conditions in a Highly-buffered Medium at Different Initial COD:Solids Ratios

Experiments were conducted at two different initial COD:biological solids ratios on acetic acid systems at an initial pH of 6.7. The sludge used in these experiments was derived from a stock reactor which was acclimated to a feed of 2000 mg/l acetic acid with an initial pH of 6.7.

Figure 20 (Experiment II) shows the response of the systems with initial COD:solids ratios of 9.9 and 8.6, respectively, under growing and nonproliferating conditions. Whereas COD removal and solids growth were exponential (first order, increasing rate) in the presence of exogenous nitrogen, COD followed decreasing first order kinetics in the nonproliferating system (Figure 21).

Maximum accumulation of intermediary compounds occurred in both systems during the first hour of metabolism of acetic acid. During this period, 36 per cent of the initial COD was converted into metabolic intermediates by the growing cells, and 50 per cent by the nonproliferating cells. It is interesting to note that no further removal of acetic acid was accomplished in the latter system until most of the intermediates accumulated earlier were metabolized.

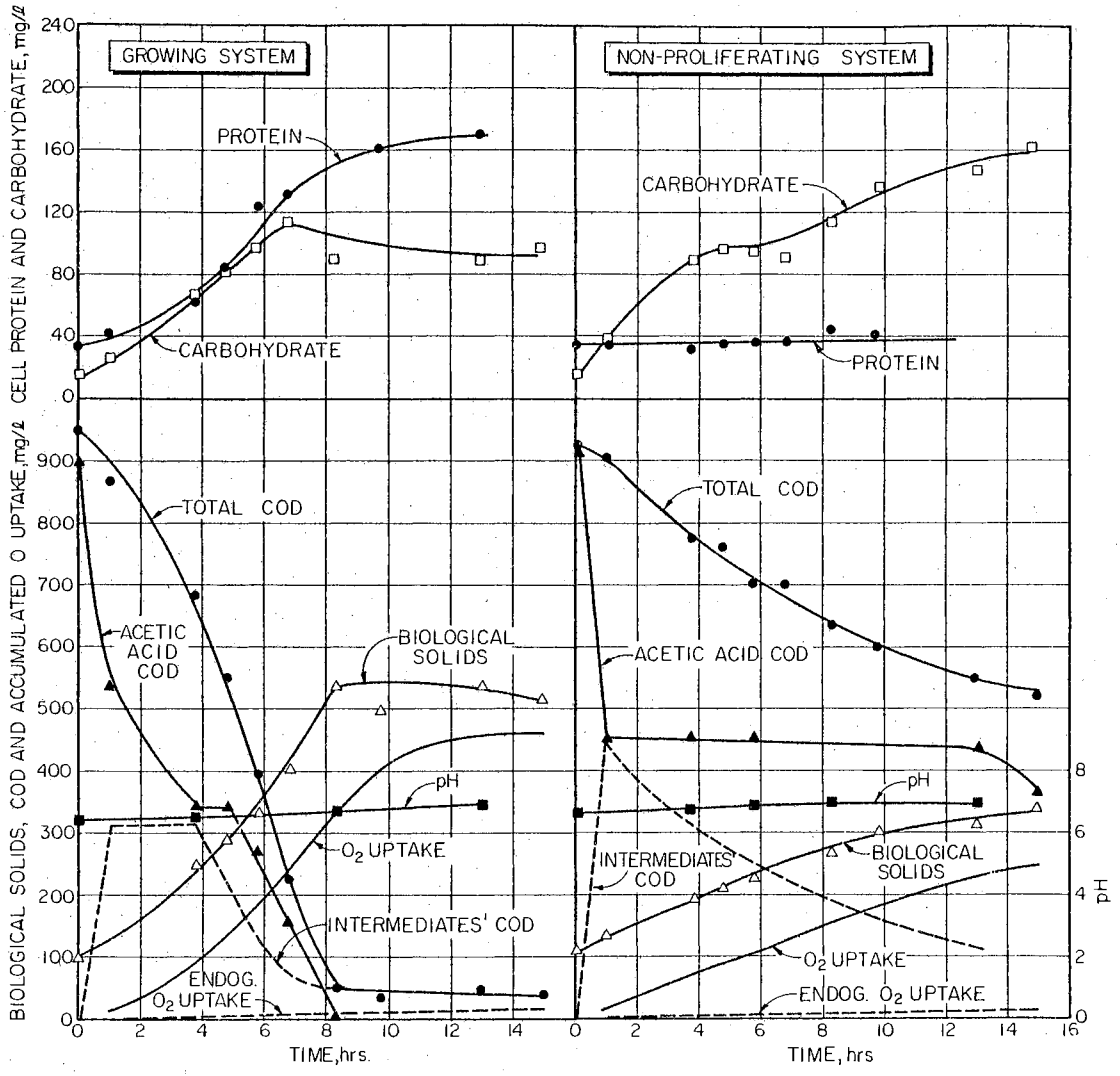


Figure 20 - Metabolism of acetic acid under growing and nonproliferating conditions in a highly buffered medium (Experiment II).

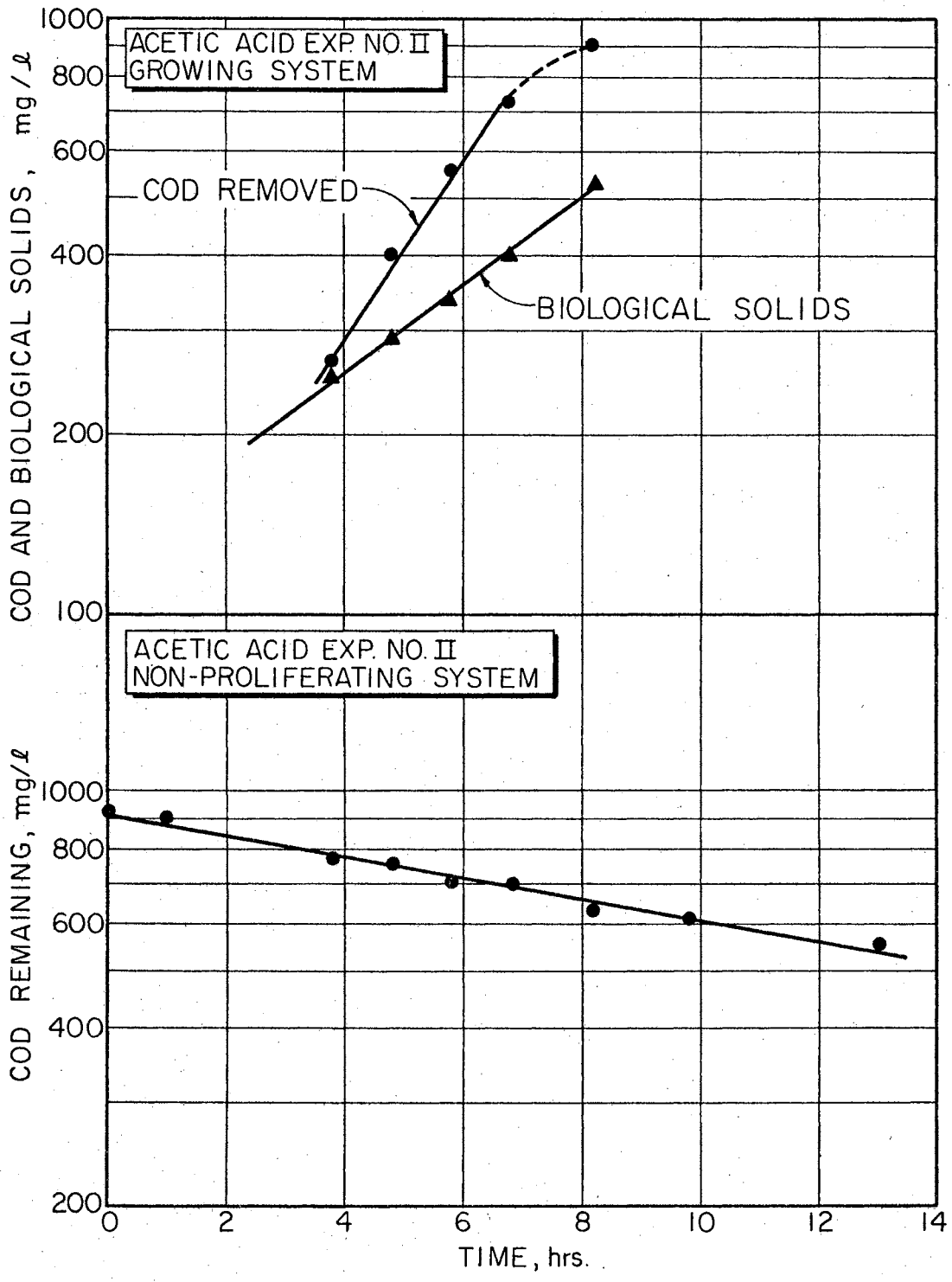


Figure 21 - Semi-log plot of COD removal and solids growth in systems shown in Figure 20.

Carbohydrate accumulation in each system is comparable to the earlier experiments at lower initial pH. Twenty-one per cent of the solids accumulated in the growth system was due to the synthesis of carbohydrate. Under nonproliferating conditions, carbohydrate accounted for 66 per cent of the net increase in solids. The net amount of carbohydrate synthesized by the cells was one and one-half times their initial dry weight. The nonproliferating cells had a high content (49 per cent) of carbohydrate as compared to the growing cells which contained only 20 per cent at the time of maximum removal of exogenous substrate. Having been synthesized in relatively small amounts, carbohydrate did not serve as an endogenous source for synthesis of nitrogenous compounds in the growing system.

At the time of maximum substrate removal, 36 per cent of the COD removed was accounted for as oxidized under growth conditions. Under nonproliferating conditions, where only 44 per cent of the initial COD was removed during the 15 hours of aeration, 62 per cent of initial COD was oxidized. The pH of both systems increased from 6.65 to 7.0 during the course of the experiment. Table XIV shows the materials balance calculations for both systems. A rather high substrate recovery is noted in the nonproliferating system.

Figure 22 shows the response of the acetic acid systems with initial COD:biological solids ratios of 3.6 and 3.3 under growing and nonproliferating conditions, respectively. The initial pH of both systems was 6.7, the same as in the previous experiment.

COD removal and solids growth were linear in the growing system. A cell yield of 35 per cent was obtained. Under nonproliferating conditions, the same kinetic order of substrate removal was noted during

TABLE XIV

MATERIALS BALANCE FOR METABOLISM OF ACETIC ACID (Exp. No. II)

Time Elapsed Hrs:Min	COD mg/l	Accumulated Decrease in COD, mg/l	COD* Calculated as Substrate mg/l	Solids mg/l	Accumulated Increase in Solids mg/l	Accumulated O_2 Uptake mg/l	Substrate Respired* mg/l	Percent Recovery
				Growing System				
00.00	950	-	-	96	-	-	-	-
01.00	870	80	75	-	-	15	14	-
03.45	684	266	250	248	152	90	84	94
04.45	550	400	375	285	189	135	127	84
05.45	395	555	521	334	238	185	174	79
06.45	226	724	679	400	304	240	225	78
08.15	49	901	845	535	439	330	310	89
09.45	35	915	858	490	394	400	375	90
13.00	49	901	845	535	439	455	427	102
15.00	42	908	852	515	419	462	432	100
				Nonproliferating System				
00.00	925	-	-	108	-	-	-	-
01.00	907	18	17	133	25	15	14	-
03.45	775	150	141	194	86	70	66	108
04.45	762	163	153	207	99	90	84	120
05.45	700	225	211	224	116	105	98	101
08.15	634	291	273	266	158	155	145	111
09.45	605	320	300	303	195	180	169	121
13.00	550	375	352	308	200	230	216	118
15.00	520	405	380	340	232	250	234	115

*Expressed as equivalent acetic acid.

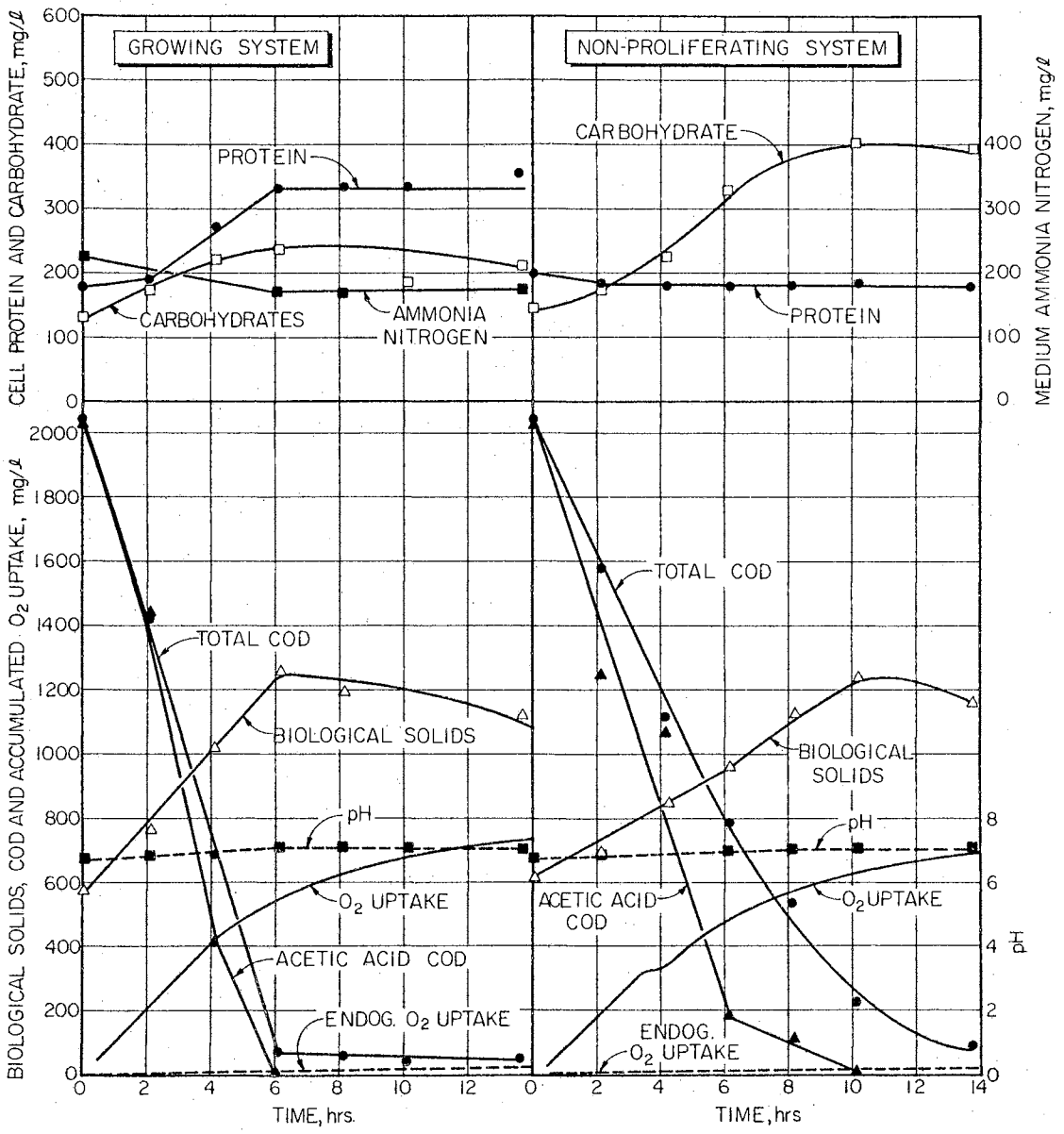


Figure 22 - Metabolism of acetic acid under growing and nonproliferating conditions in a highly buffered medium (Experiment III).

the first six hours of operation; this was, however, subsequently followed by a decreasing reaction rate. Solids concentration was maximum at the end of ten hours in the nonproliferating system, even though the medium still contained 11 per cent of the initial COD. Further COD removal did not elicit any response in solids level which, in fact, started to decline. A cell yield of 34 per cent was obtained.

Between two and six hours of operation a maximum of 278 mg/l of COD (13 per cent of the initial COD) was present in the form of metabolic intermediates in the growing system. These intermediates were subsequently utilized at the same rate as the substrate. The residual COD at the end of the experiment (13 hours and 40 minutes) was 56 mg/l. Substrate removal was much faster during the first six hours in the nonproliferating system than it was in the later period. A maximum of 621 mg/l of COD (30 per cent of initial COD) accumulated as metabolic intermediates during the first six hours. The rate of utilization of intermediates by the cells was slower than the COD removal rate of the early phase. The residual COD at the end of the experiment was 96 mg/l.

The growth system did not exhibit an ability to accumulate carbohydrate as the primary endogenous reserve material. Only 15 per cent of the solids synthesized under growth conditions could be accounted for as carbohydrate. The carbohydrate content of the cells dropped from 23 to 18 per cent during the substrate removal phase. Under nonproliferating conditions, 42 per cent of the solid accumulation was due to carbohydrate. Cell carbohydrate increased from 23 to 33 per cent during the substrate removal phase. It is interesting to note that carbohydrate did not serve as the endogenous carbon source for the synthesis of protein in the growth system.

At the time of maximum COD removal (96 per cent of the initial value) and solids growth (about six hours), 28 per cent of the COD removed was accounted for as oxidized under growth conditions. However, under nonproliferating conditions, 35 per cent of the COD removed was oxidized at the time of maximum solids accumulation (about ten hours), when 89 per cent of the initial COD had been removed.

Materials balances for these systems are given in Table XV. It is seen that the percent recovery was uniformly low in both systems. This suggests that a part of the substrate might have been converted into volatile intermediary products and stripped from the system.

b. Metabolism of Sodium Acetate

i) Experiment I. Metabolism of Sodium Acetate Under Growing and Nonproliferating Conditions in the Normal Growth Medium

Figure 23 shows the response of the growing and nonproliferating systems with an initial COD:biological solids ratio of 4.7 and an initial pH of 7.2. The buffering capacity of the medium was the same as that employed in the normal growth medium (Table I).

The pH of the medium in the growing system continually increased during the removal of acetate from the medium. Substrate removal proceeded at a lower rate than even in the acetic acid systems with higher COD:biological solids ratios (Figures 18 and 20). At the termination of the experiment (17 hours), 15 per cent of the initial COD was still present in the medium; two-thirds of this amount was in the form of metabolic intermediates. Thirty-five per cent of the theoretical O_2 demand had been exerted up to this time.

Growth was severely restricted in the absence of nitrogen, and

TABLE XV

MATERIALS BALANCE FOR METABOLISM OF ACETIC ACID (Exp. No. III)

Time Elapsed Hrs:Min	COD mg/l	Accumulated Decrease in COD, mg/l	COD* Calculated as Substrate mg/l	Solids mg/l	Accumulated Increase in Solids mg/l	Accumulated O_2 Uptake mg/l	Substrate Respired* mg/l	Percent Recovery
				Growing System				
00.00	2040	-	-	570	-	-	-	-
02.10	1420	620	582	750	180	230	216	68
04.10	688	1352	1268	1020	450	420	394	67
06.10	72	1968	1846	1250	680	550	516	65
08.10	64	1976	1853	1185	615	630	591	65
10.10	48	1992	1868	-	-	-	-	-
13.40	56	1984	1861	1115	545	735	689	66
				Nonproliferating System				
00.00	2040	-	-	610	-	-	-	-
02.10	1580	460	431	690	80	200	188	62
04.10	1120	920	863	840	230	350	328	65
06.10	792	1248	1171	960	350	480	450	68
08.10	536	1504	1411	1120	510	575	539	74
10.10	232	1808	1696	1230	620	635	596	72
13.40	96	1944	1823	1165	555	690	647	66

*Expressed as equivalent acetic acid.

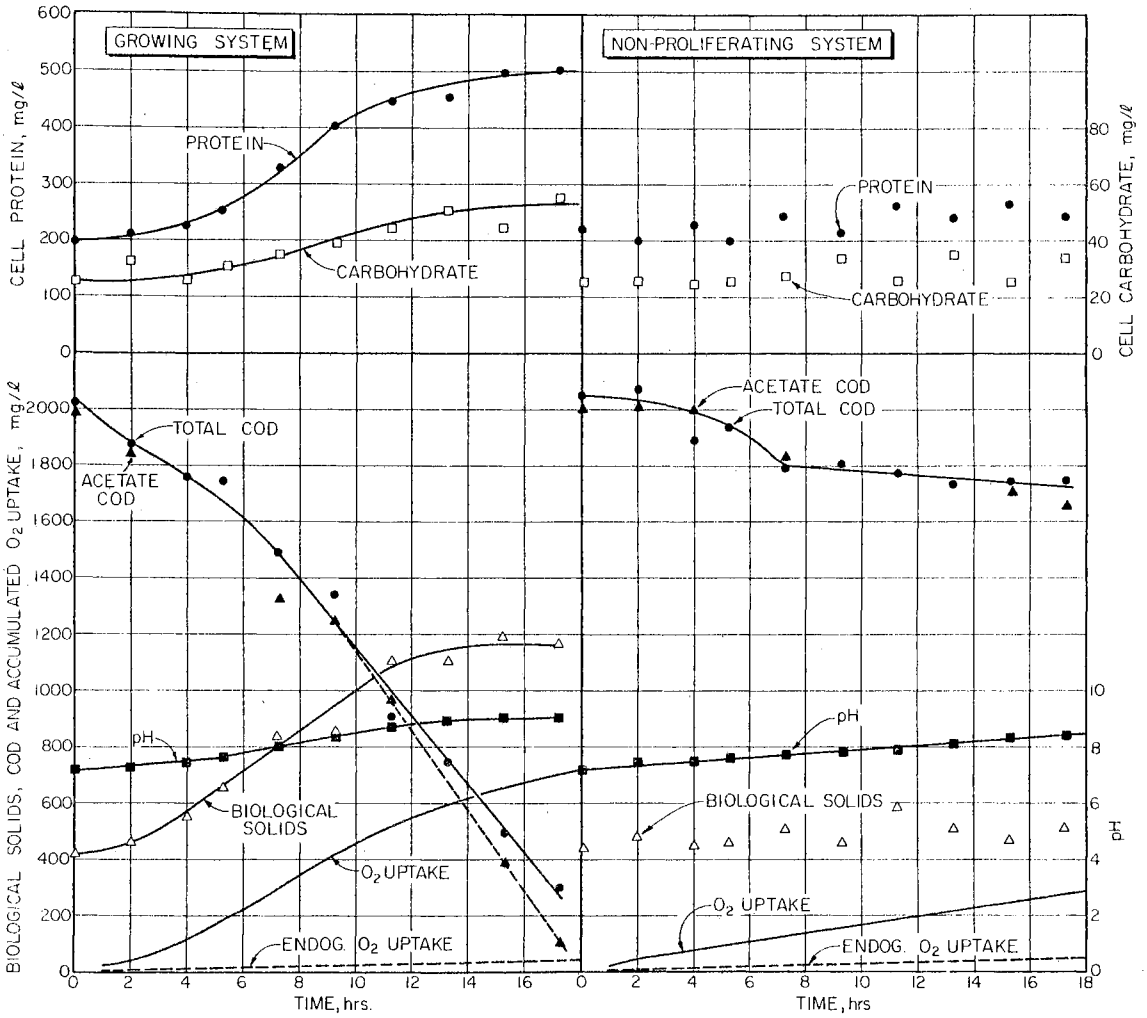


Figure 23 - Metabolism of sodium acetate under growing and nonproliferating conditions in a normal growth medium (Experiment I).

only 15 per cent of the initial COD was metabolized during 17 hours of aeration. Most of the COD removed could be accounted for as oxidized. No metabolic intermediates accumulated in this system.

The pH of the medium had increased from 7.2 to 9.0 in the growing system, and to 8.4 in the nonproliferating system at the time the experiments were terminated. The carbohydrate content of the cells in both systems varied from 4 to 7 per cent of dry weight during the entire course of the experiment. Synthesis of protein and carbohydrate contributed 42 per cent and 4 per cent, respectively, of the solids synthesized in the growing system. Table XVI shows the materials balances for these systems.

ii) Experiment II. Metabolism of Sodium Acetate Under Growing and Nonproliferating Conditions in a Highly-buffered Medium

Figure 24 shows the response of the cells acclimated to a growth medium with 1000 mg/l sodium acetate as the carbon source, and 40 ml/l of 1.0 M phosphate buffer.

Growth and COD removal were exponential (first order, increasing rate) in the presence of nitrogen. Decreasing first order kinetics were observed in the nonproliferating system where the cell yield was only half of that obtained in the growing system. However, respiration was higher in the nonproliferating system. As much as 47 per cent of the COD removed by the growing cells was accounted for as oxidized at the time of 97 per cent COD removal (5 hours and 45 minutes). Fifty-six per cent of the initial COD was accounted for as oxidized in the nonproliferating system at the time of 88 per cent COD removal (9 hours and 15 minutes).

Very little carbohydrate was synthesized in either system. It

TABLE XVI

MATERIALS BALANCE FOR METABOLISM OF SODIUM ACETATE (Exp. No.I)

Time Elapsed Hrs:Min	COD mg/l	Accumulated Decrease in COD, mg/l	COD* Calculated as Substrate mg/l	Solids mg/l	Accumulated Increase in Solids mg/l	Accumulated O_2 Uptake mg/l	Substrate Respired* mg/l	Percent Recovery
				Growing System				
00.00	2000	-	-	425	-	-	-	-
02.00	1880	120	113	462	37	41	39	67
04.00	1760	240	226	550	125	120	113	61
05.15	-	-	-	657	232	173	163	-
07.15	1490	510	481	832	407	300	283	143
09.15	1335	665	627	845	420	417	393	130
11.15	908	1092	1030	1100	675	515	486	113
13.15	745	1255	1184	1105	680	590	556	104
15.15	490	1510	1425	1188	763	660	622	97
17.15	300	1700	1604	1155	730	705	665	87
				Nonproliferating System				
00.00	2050	-	-	440	-	-	-	-
02.00	2070	-	-	478	38	40	38	-
04.00	1890	160	151	450	10	75	71	54
05.15	1945	105	99	457	17	97	92	110
07.15	1795	255	241	505	65	130	123	78
09.15	1816	234	221	460	20	160	151	77
11.15	1780	270	255	587	147	190	179	128
13.15	1725	325	307	510	70	220	208	91
15.15	1740	310	292	467	27	245	231	88
17.15	1740	310	292	517	77	270	255	114

*Expressed as equivalent acetic acid.

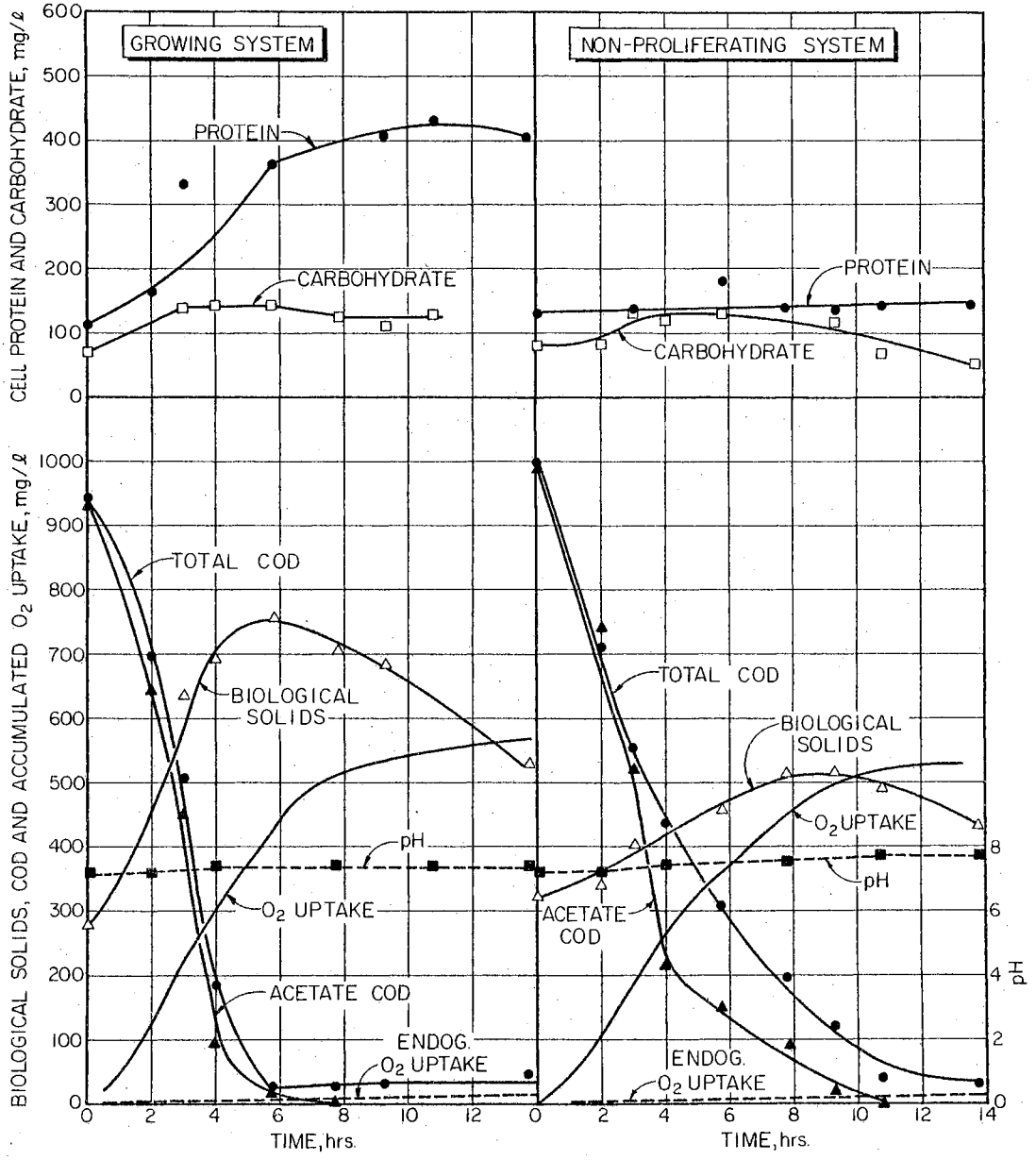


Figure 24 - Metabolism of sodium acetate under growing and nonproliferating conditions in a highly buffered medium (Experiment II).

accounted for only 16 and 21 per cent of the solids accumulated in the growing and nonproliferating systems, respectively. Nitrogen limitation did not seem to promote the storage of cellular carbohydrate. In contrast to the system reported earlier, where the pH of the medium increased during growth, the cells in the present experiment had a higher content (25 per cent dry weight) of carbohydrate. Also synthesis of protein was much higher (52 per cent of net increase in solids) in the growing system.

Maximum accumulation of metabolic intermediates amounted to ten per cent of the initial COD in the growing system, and 22 per cent in the nonproliferating system. These were subsequently utilized in both systems. There was no significant difference in the residual COD between the two systems. The pH increased from an initial value of 7.2 to 7.5 in the growing system, and from 7.2 to 7.7 in the nonproliferating system.

Materials balances for these systems are presented in Table XVII. Excellent substrate recoveries were obtained for the growing system.

c. Metabolism of Propionic Acid

i) Experiment I. Metabolism of Propionic Acid Under Growing and Nonproliferating Conditions in a Medium Deficient in Buffering Capacity

Figure 25 shows the response of the growing system with an initial COD:biological solids ratio of 15 and an initial pH of 4.5. The cells used in this study were acclimated to a growth medium containing 2000 mg/l of propionic acid (3000 mg/l propionic COD) as carbon source and 20 ml/l of 1.0 M phosphate buffer. Calculated on a buffer:COD basis, the concentration of buffer employed in this study is slightly less than normally used.

TABLE XVII

MATERIALS BALANCE FOR METABOLISM OF SODIUM ACETATE (Exp. No.II)

Time Elapsed Hrs:Min	COD mg/l	Accumulated Decrease in COD, mg/l	COD* Calculated as Substrate mg/l	Solids mg/l	Accumulated Increase in Solids mg/l	Accumulated O_2 Uptake mg/l	Substrate Respired* mg/l	Percent Recovery
				Growing System				
00.00	940	-	-	275	-	-	-	-
02.00	696	244	229	-	-	120	113	139
03.00	507	433	406	635	360	220	206	98
04.00	183	757	710	690	415	300	281	103
05.45	27	913	856	755	480	425	399	105
07.45	27	913	856	700	425	510	478	106
09.15	34	906	850	682	407	530	497	108
13.45	48	892	837	527	252	-	-	-
				Nonproliferating System				
00.00	1000	-	-	320	-	-	-	-
02.00	712	288	270	332	12	110	103	43
03.00	555	445	417	405	85	185	174	62
04.00	433	567	532	-	-	265	249	-
05.45	312	688	645	455	135	360	338	89
07.45	193	807	757	517	197	450	422	82
09.15	122	878	824	515	193	495	464	80
10.45	41	959	900	485	165	520	488	73
13.45	31	969	909	430	110	-	-	-

*Expressed as equivalent acetic acid.

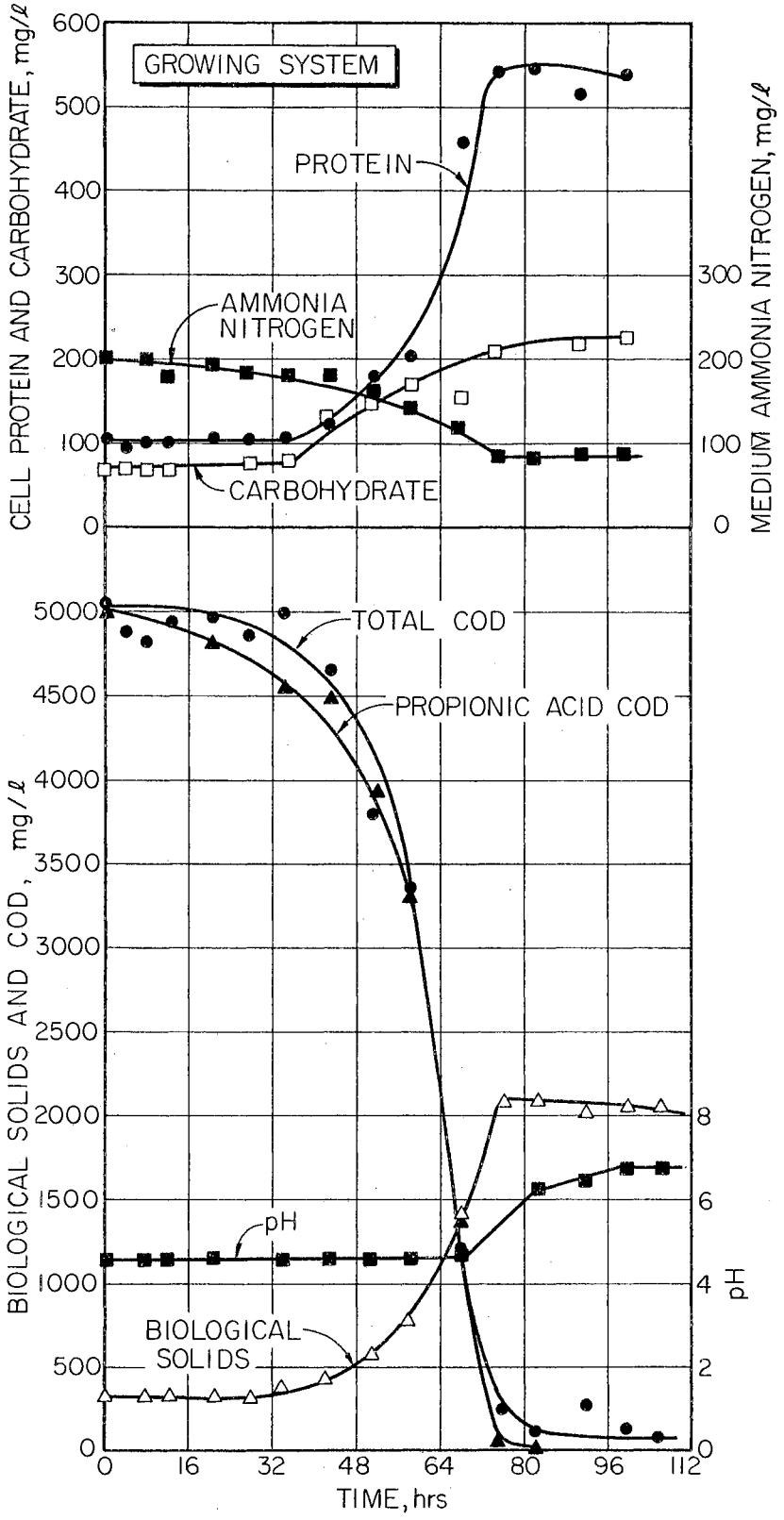


Figure 25 - Metabolism of propionic acid under growing conditions in a buffer-deficient medium (Experiment I).

There was a lag extending for a period of two days before cell growth was initiated. After this prolonged lag, solids growth and COD removal were exponential (Figure 26). The pH of the medium remained below 5.0 throughout the major part of the substrate removal phase. The nonproliferating system with comparable initial solids and pH failed to support growth during a prolonged period of observation (more than 100 hours). Small amounts of metabolic intermediates (amounting to 3.5 per cent of the initial COD) accumulated during the transition from the lag to the exponential phase in the growing system. These were subsequently utilized by the cells.

Only eight per cent of the net solids accumulation was due to synthesis of carbohydrate. At the time of substrate removal (76 hours) the cells contained only 10 per cent carbohydrate. Protein synthesis which progressed during exponential growth amounted to only 25 per cent of the net increase in solids.

ii) Experiment II. Metabolism of Propionic Acid Under Growing and Nonproliferating Conditions in an Adequately-buffered Medium

Figure 27 shows the metabolic response of the propionic acid systems with an initial COD:biological solids ratio of 5, and an initial pH of 5.3. Twenty ml of 1.0 M phosphate buffer per 1000 mg propionic acid per liter was used in the growth medium of the cells employed in this experiment.

No lag in growth was observed in these experiments. Cell growth was exponential (first order, increasing rate) in the growing system (Figure 28). COD removal followed decreasing first order reaction kinetics in the nonproliferating system. The cell yields were 40 and 35 per cent, respectively, under growing and nonproliferating

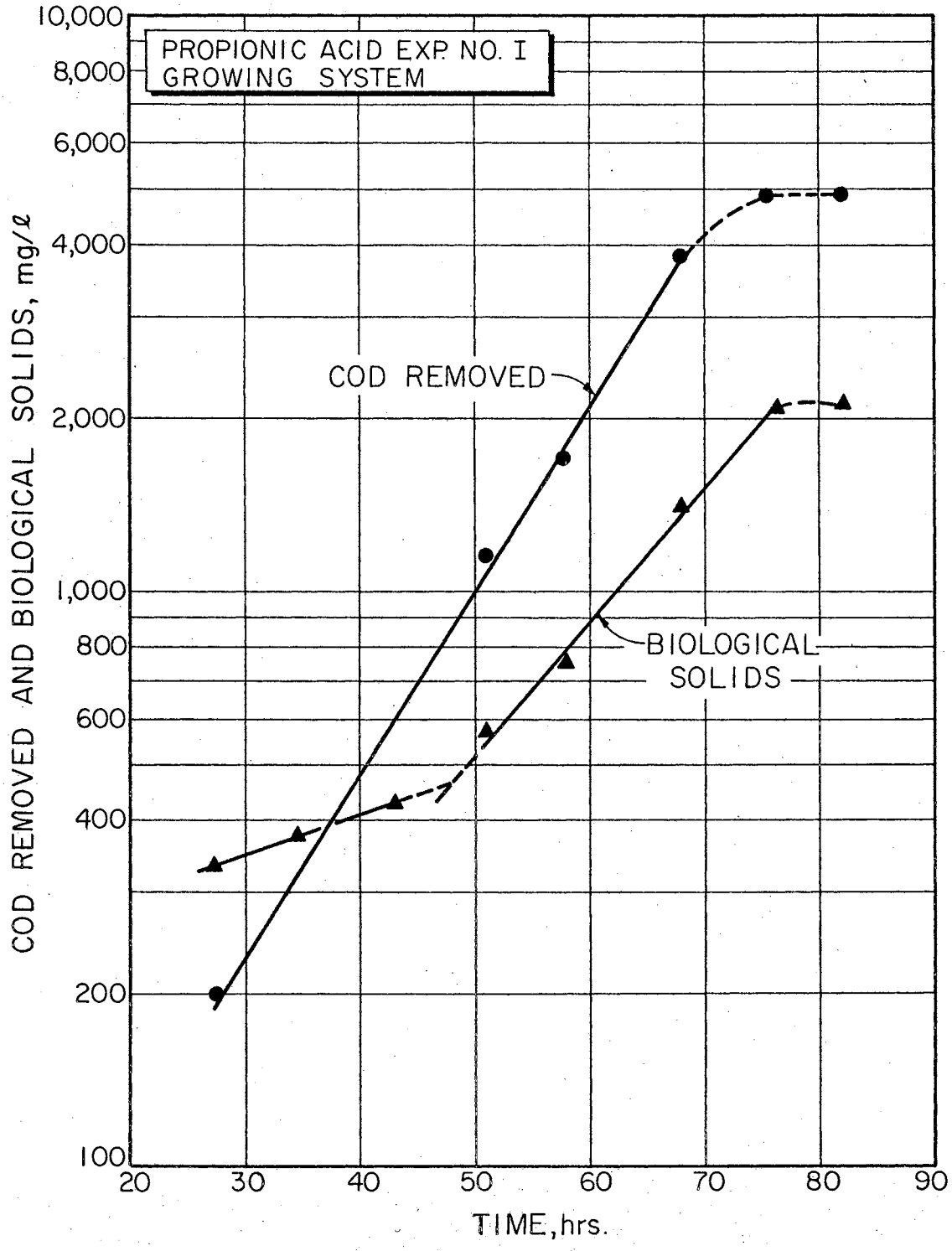


Figure 26 - Semi-log plot of COD removal and solids growth in system shown in Figure 25.

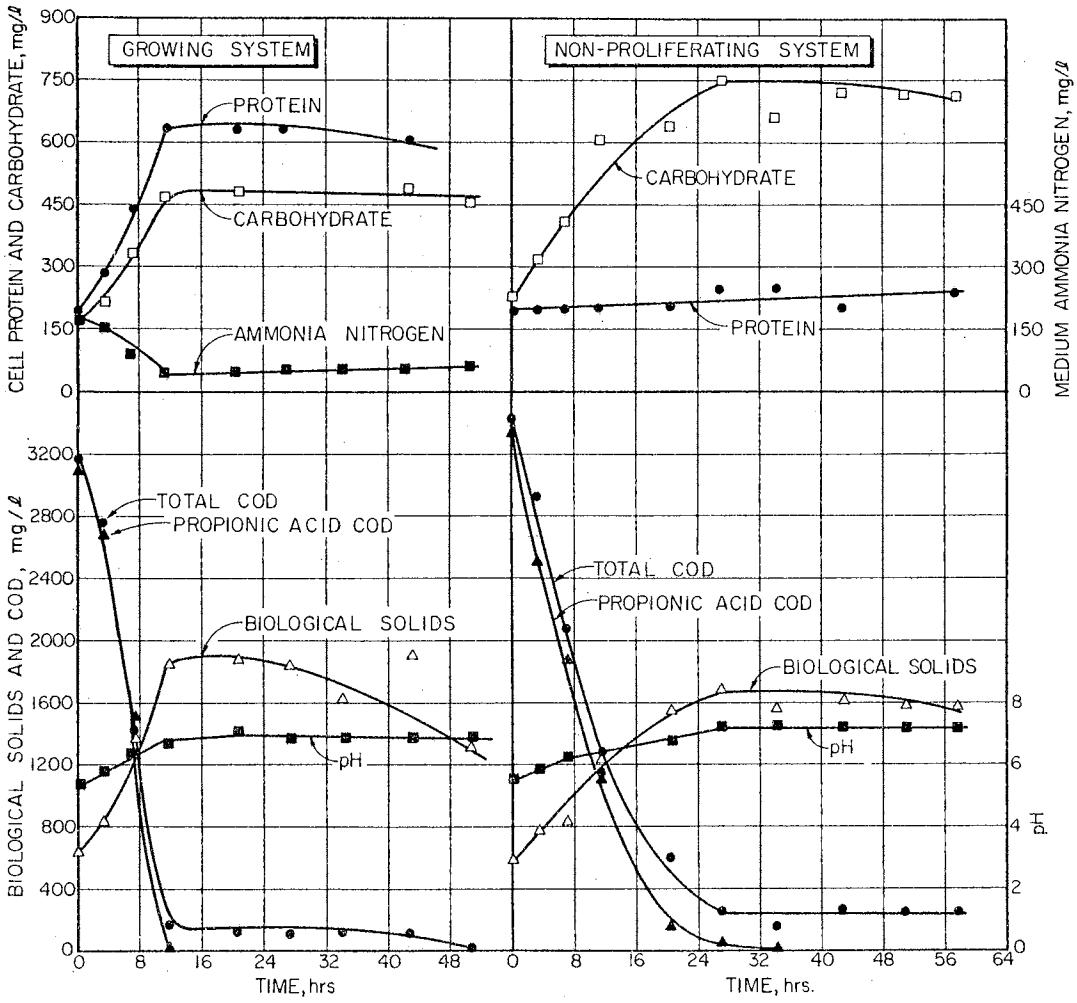


Figure 27 - Metabolism of propionic acid under growing and nonproliferating conditions in an adequately buffered medium (Experiment II).

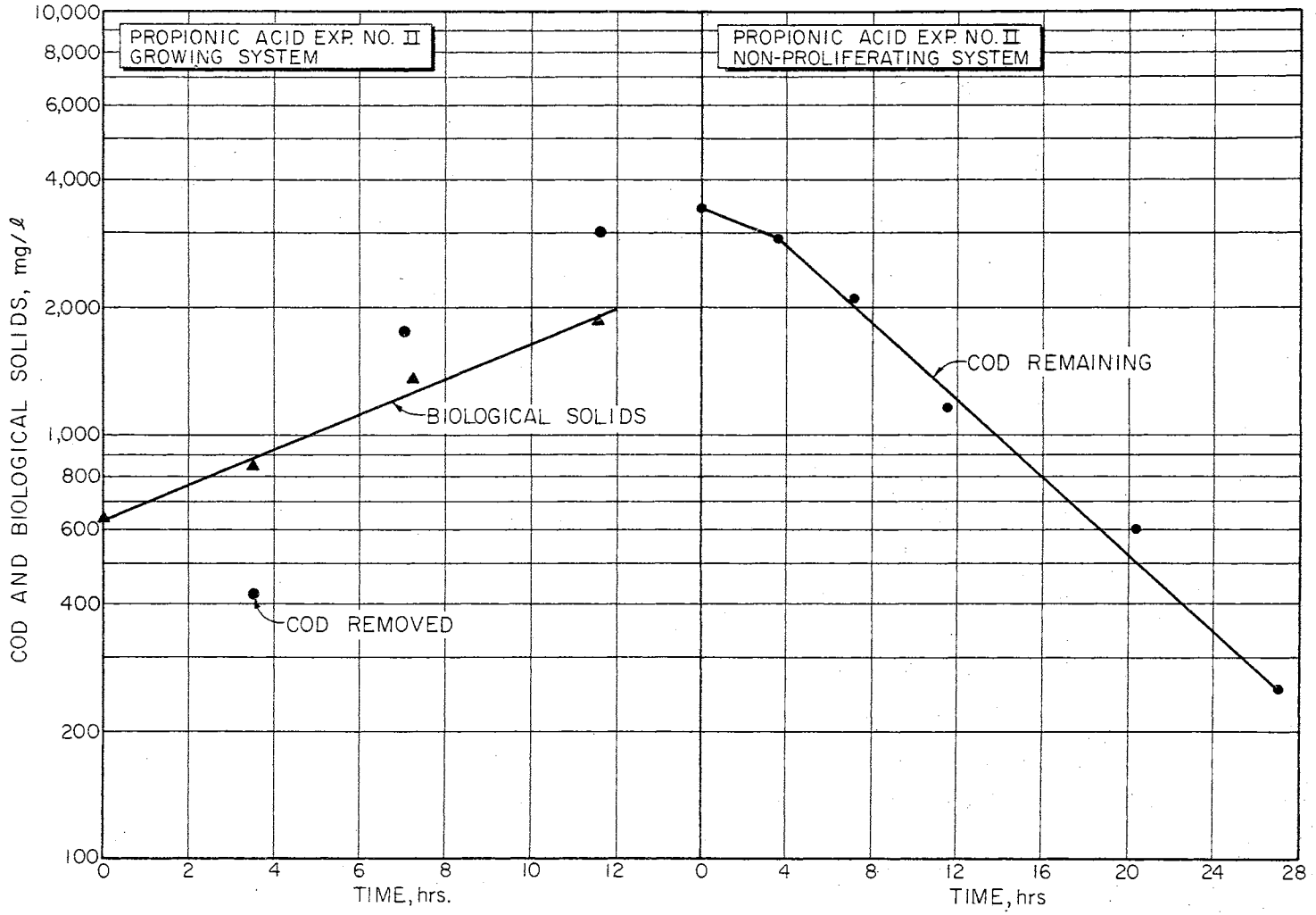


Figure 28 - Semi-log plot of COD removal and solids growth in systems shown in Figure 27.

conditions. Maximum accumulation of metabolic intermediates amounted to only five per cent of the initial COD in the growth system, and 13 per cent in the nonproliferating system. The metabolic intermediates released by the nonproliferating cells were found to be anthrone-reactive. No attempt was made to identify the carbohydrates. Almost half of the amount of total intermediates released by the cells in the nonproliferating system remained in the medium as endproducts at the termination of the experiment (57 hours and 40 minutes). Almost no COD was detected in the medium of the growing system at the end of 51 hours.

Synthesis of both protein and carbohydrate peaked at the time of maximum COD removal in the growing system. The growing cells in the present experiment accumulated larger amounts of carbohydrate and protein than did those in the previous experiment. Twice as much carbohydrate was synthesized by the nonproliferating cells as by the growing cells. At the time of termination of the experiment the cells in the nonproliferating system contained 46 per cent carbohydrate and 15 per cent protein, and the corresponding figures for the cells in the growing system were 35 and 48. The growing cells had consumed 133 mg/l of ammonia nitrogen from the medium. No ammonia nitrogen was detected in the medium of the nonproliferating system, and organic-nitrogen was absent in the medium of both systems.

The pH of the growth system rose from an initial value of 5.3 to 6.7 at the time of maximum substrate removal. Under nonproliferating conditions the pH increased from 5.5 to 7.1 during the corresponding period.

C. Long-term Batch Studies on the Solids Accumulation and Endogenous Phases of Growing and Nonproliferating Systems

The short-term batch experiments reported earlier had indicated some correlation between the concentration of initial biological solids and the production of metabolic intermediates and/or endproducts. It was also noted that total endogenous oxidation of cellular carbohydrate can occur under growth conditions (Figure 15). In the light of these findings, a systematic study of the effect of initial biological solids concentration and nitrogen supply on metabolic patterns during the substrate removal and endogenous phases was undertaken.

Two polyhydric alcohols, glycerol and sorbitol, were chosen as the substrates for this study. Glycerol was chosen because of its close relationship to the triose phosphate stage of glycolysis. Since the cells grown with fatty acids showed a somewhat reduced capacity to synthesize carbohydrate, it was considered desirable to ascertain whether the reversal of glycolysis can occur from the triose level.

1. Metabolism of Glycerol

Experiments at three different initial COD:biological solids ratios were conducted using cells from the same stock reactor. Table XVIII shows the outline and duration of the experiments.

a. Experiment I. Metabolism of Glycerol by Growing and Nonproliferating Cells at High Initial COD:Biological Solids Ratio

Figure 29 shows the changes in parameters measured during the metabolism of glycerol at an initial COD:biological solids ratio of 21.

i) Solids Accumulation Phase

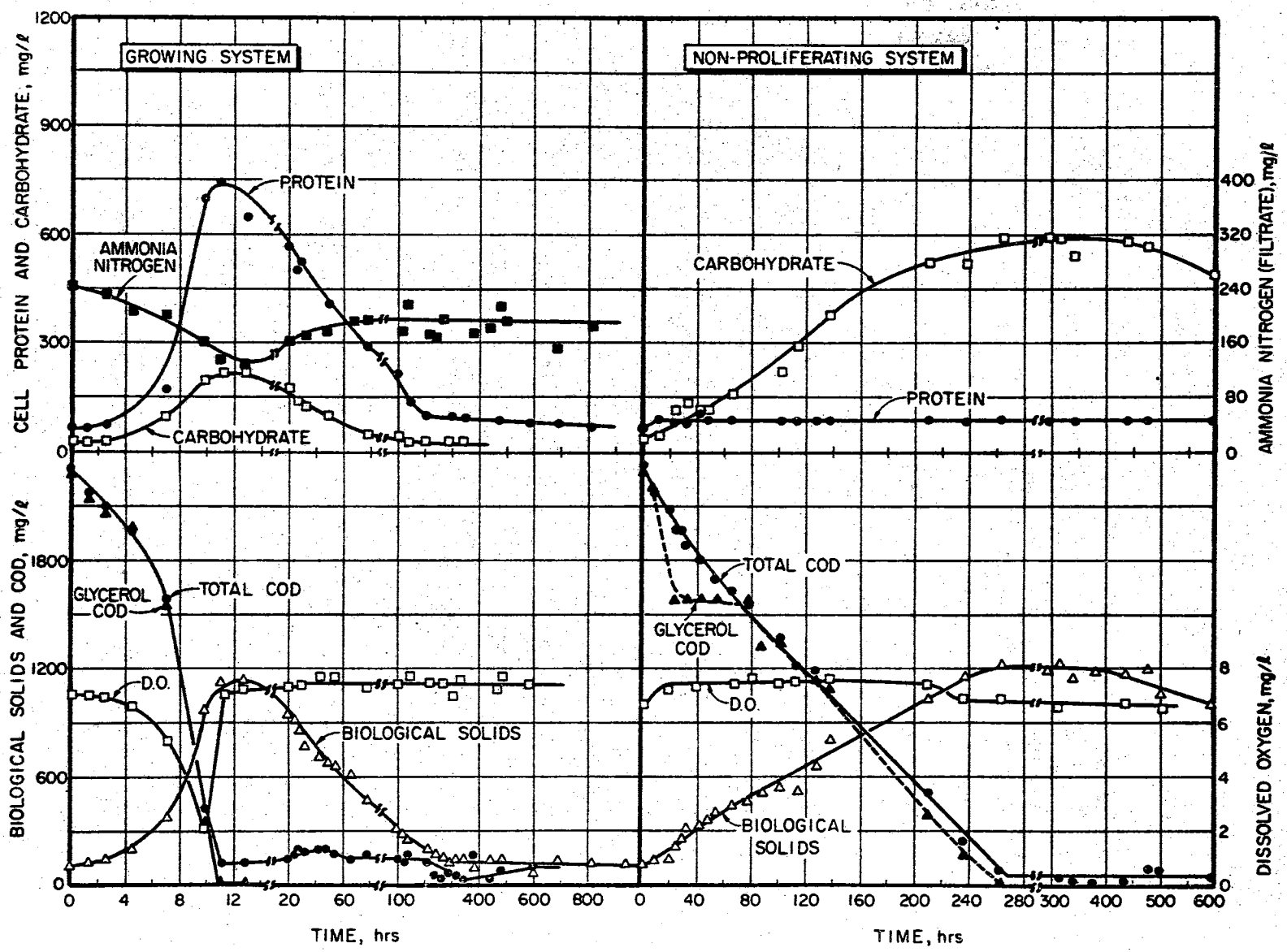
The substrate utilization patterns were drastically different in the growing and nonproliferating systems. COD removal and solids growth were exponential during the entire period of glycerol utilization

TABLE XVIII

EXPERIMENTAL OUTLINE FOR LONG-TERM BATCH EXPERIMENTS ON GLYCEROL

Exp. No.	Growing System				Nonproliferating System			
	Initial COD mg/l	Initial Biol. Solids mg/l	Initial COD / Initial Biol. Solids	Duration hrs	Initial COD mg/l	Initial Biol. Solids mg/l	Initial COD / Initial Biol. Solids	Duration hrs
I	2280	110	20.7	934	2300	112	20.6	595
II	2340	283	8.3	416	2240	318	7.0	416
III	3430	1110	3.1	273	3370	1088	3.1	273

Figure 29 - Comparison of solids accumulation and endogenous phases under growing and nonproliferating conditions during metabolism of glycerol at high initial COD:biological solids ratio (Experiment I).


GROWING SYSTEM
NON-PROLIFERATING SYSTEM
CELL PROTEIN AND CARBOHYDRATE, mg/ℓ
AMMONIA NITROGEN (FILTRATE), mg/ℓ
BIOLOGICAL SOLIDS AND COD, mg/ℓ
DISSOLVED OXYGEN, mg/ℓ
PROTEIN
AMMONIA NITROGEN
CARBOHYDRATE
GLYCEROL COD
TOTAL COD
D.O.
BIOLOGICAL SOLIDS
TIME, hrs
TIME, hrs

in the growth system. Under nonproliferating conditions, a semi-log plot of the COD remaining in the system (Figure 30) showed at least two phases of decreasing first-order kinetics having different rates. Similarly, solids accumulation occurred at a decreasing rate. Whereas only 11 hours were required for the elimination of substrate from the medium under growth conditions, it took more than 260 hours in the system devoid of nitrogen. The cell yields were comparable in the two systems.

The nature and amounts of metabolic intermediates and endproducts released by the cells under both conditions are shown in Table XIX. No significant amounts of non-glycerol compounds were released by the growing cells during the substrate removal period. Small amounts of acetic acid (5 to 8 mg/l) and ribose (2 to 4 mg/l) were detected in the medium in the late log growth phase. However, under nonproliferating conditions as much as 20 per cent of the initial COD was converted into metabolic intermediates and endproducts during the first 24 hours of glycerol metabolism. These intermediates were subsequently utilized during the next 60 hours. The quantities of acetic acid (26 mg/l), keto acids (2 to 5 mg/l), and ribose (2 to 7 mg/l) detected in the system were slightly higher than those observed in the growth system.

Synthesis of protein predominated throughout the substrate removal phase in the growing system, accounting for 69 per cent of solids synthesized. Carbohydrate constituted only 19 per cent of the increase in cell mass. In the nonproliferating system more than 50 per cent of the solids accumulation was due to the synthesis of carbohydrate. At the time of maximum solids synthesis, protein content of the cells in the growing and nonproliferating systems were 68 per cent and 7 per cent,

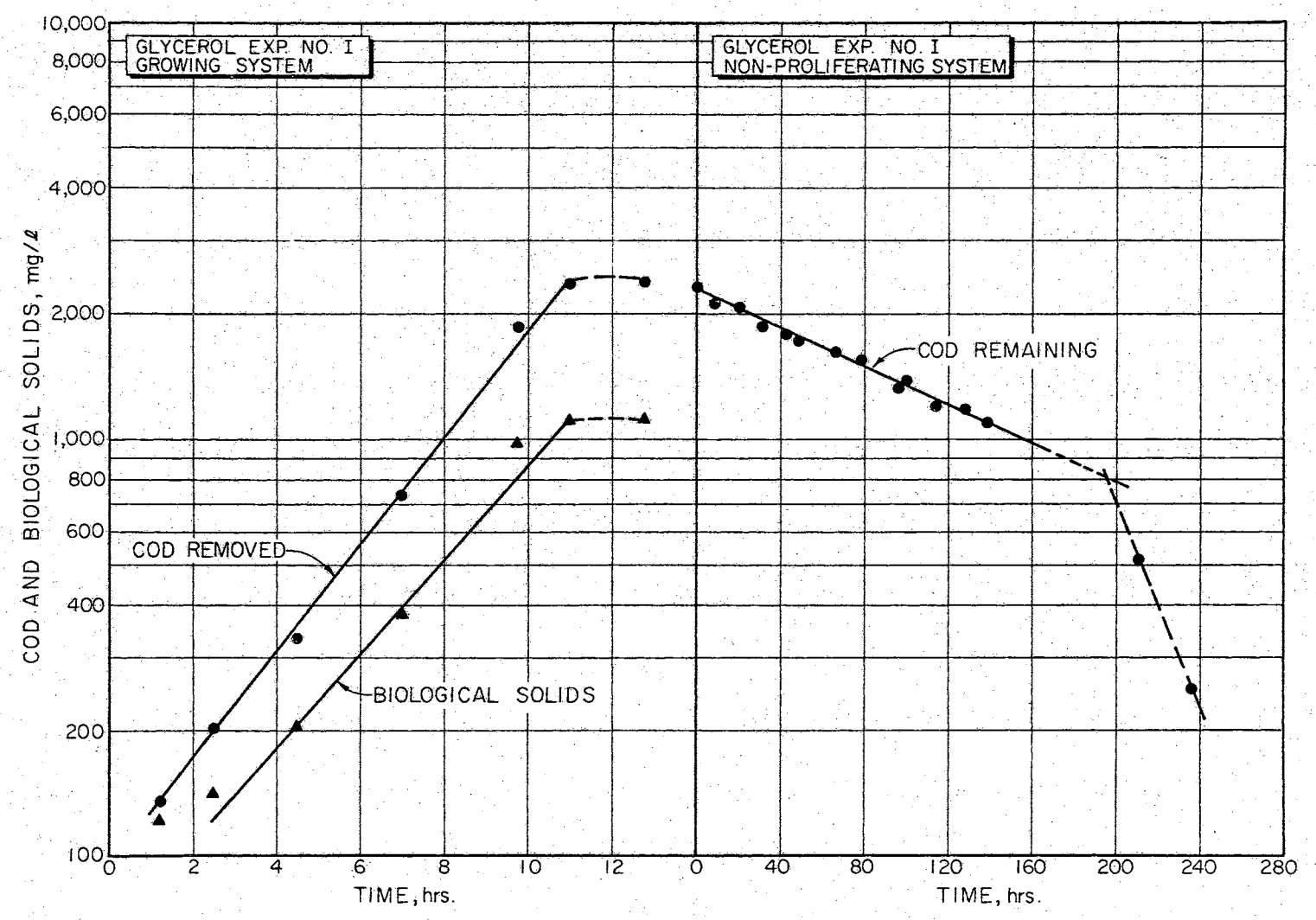


Figure 30 - Semi-log plot of COD removal and solids growth in systems shown in Figure 29.

TABLE XIX

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF GLYCEROL (Exp. No. I)

Time Elapsed Hrs:Min	Intermediates and/or Endproducts - COD, mg/l							
	Growing System				Nonproliferating System			
	Total	Acetic Acid	Ribose	Keto Acids	Total	Acetic Acid	Ribose	Keto Acids
07.00	20	8	trace	-	nil	-	-	-
09.45	75	5	2	2	-	-	-	-
*11.00	117	nil	4	-	-	-	-	-
12.45	125	"	4	trace	-	nil	-	5
19.45	153	"	10	-	-	"	2	4
23.30	169	"	11	nil	410	"	2	3
27.30	200	"	14	"	-	"	2	3
31.00	200	"	10	trace	290	"	2	3
32.25	192	"	16	"	310	"	1	3
42.05	200	"	-	"	215	"	2	3
47.50	201	"	16	"	-	"	-	3
54.00	175	"	-	"	110	"	3	3
66.00	142	"	11	"	-	"	3	-
77.30	175	"	-	"	nil	"	-	-
88.15	146	"	5	"	"	"	7	-
101.00	146	"	4	"	5	4	3	-
114.00	141	"	6	"	-	-	-	-
128.00	121	"	3	"	40	26	3	4
138.00	166	"	3	"	nil	nil	-	-
210.30	125	"	2	"	113	"	3	2
236.30	57	"	2	"	96	trace	2	-
**263.30	32	"	4	"	57	"	2	-
294.30	65	"	3	"	49	6	2	-
317.00	57	"	2	"	40	4	2	trace
340.30	16	"	3	"	16	2	2	-
381.30	179	"	2	"	16	2	2	-
436.00	32	"	-	"	24	nil	trace	-
473.30	89	"	-	"	105	"	"	-
497.45	105	"	2	"	90	"	"	trace
594.45	98	"	2	"	41	"	"	-

* Glycerol eliminated from medium in growing system
** Glycerol eliminated from medium in nonproliferating system.

respectively. On the other hand, the growing cells contained only 19 per cent carbohydrate as compared with 49 per cent in the nonproliferating cells.

ii) Endogenous Phase

Endogenous oxidation of solids was drastically different in the growing and nonproliferating systems. Almost "total oxidation" of the solids accumulated under balanced growth conditions was observed during the first 280 hours of endogenous metabolism. Carbohydrates and protein were utilized simultaneously during the endogenous phase in the growing system. Total oxidation of protein and carbohydrate accumulated during growth occurred in the endogenous phase.

The rate of endogenous oxidation was very slow in the nonproliferating system. A prolonged stationary phase occurred after the attainment of maximum solids level. Holding the solids accumulated in the absence of nitrogen for 330 hours under endogenous respiration conditions resulted in only 20 per cent oxidation of accumulated solids. It is seen from the figure that carbohydrate, which accounted for a large part of the solids synthesized, was not readily utilized during the endogenous phase.

Liberation of ammonia nitrogen by the cells in the growing system began immediately upon the onset of the endogenous phase. As much as 55 per cent of the ammonia nitrogen consumed by the cells during the growth phase was released into the medium in the endogenous phase. No organic nitrogen could be detected in the medium during the entire course of the experiment. Neither ammonia nor organic nitrogen was found in the medium of the nonproliferating system.

Significant amounts of ribose were detected during the early

endogenous phase of the growing system (see Table XIX). Ribose persisted in the medium during the entire endogenous phase of this system. Small amounts of acetic acid and ribose appeared in the medium during the initial period of endogenous metabolism in the nonproliferating system.

b. Experiment II. Metabolism of Glycerol by Growing and Nonproliferating Cells at an Intermediate Ratio of Initial COD:Initial Biological Solids

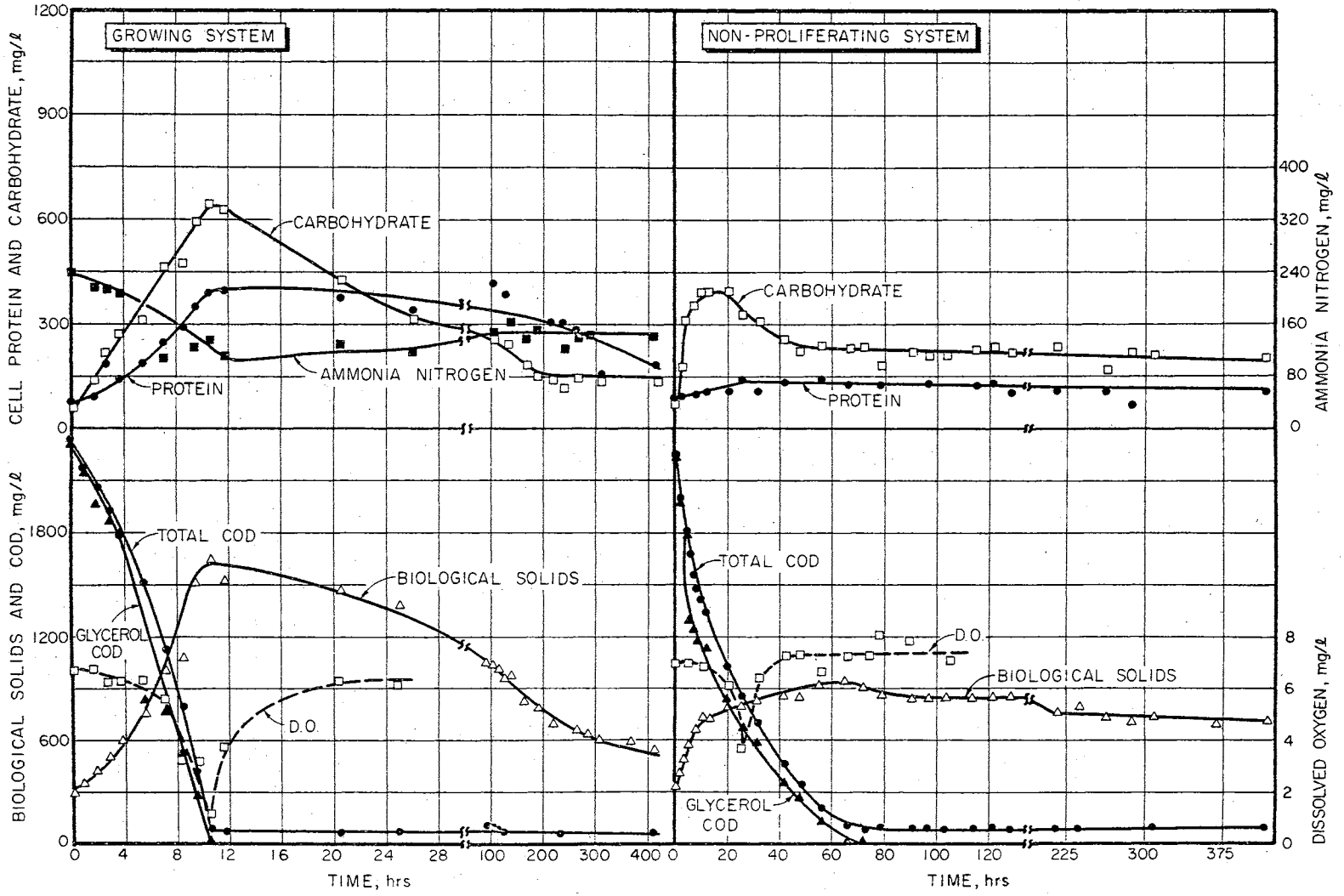
Figure 31 shows the response of the systems when the initial COD: biological solids ratio was 8 in the growing system and 7 in the nonproliferating system.

i) Solids Accumulation Phase

COD removal and solids production patterns in the growth system were similar to the growth system of Experiment I. COD removal under nonproliferating conditions consisted of a series of first-order reactions, as shown in Figure 32. Comparison of Figures 29 and 31 indicates that a great improvement in COD removal rate in nonproliferating systems can be expected with an increase in the initial biological solids concentration. However, there was still a large difference in the COD removal rates of the growing and nonproliferating systems. Except for an initial brief period of linear increase, the rate of solids accumulation was drastically reduced in the nonproliferating system. Final solids yield was less than half of that in the corresponding growth system.

Table XX shows the extracellular metabolic products released by the cells in both systems. Nonproliferating cells produced slightly larger amounts of non-glycerol COD than did the growing cells. No

Figure 31 - Comparison of solids accumulation and endogenous phases under growing and nonproliferating conditions during metabolism of glycerol at an intermediate ratio of initial COD:biological solids (Experiment II).


GROWING SYSTEM
NON-PROLIFERATING SYSTEM
CELL PROTEIN AND CARBOHYDRATE, mg/ℓ
AMMONIA NITROGEN, mg/ℓ
BIOLOGICAL SOLIDS AND COD, mg/ℓ
DISSOLVED OXYGEN, mg/ℓ
CARBOHYDRATE
PROTEIN
AMMONIA NITROGEN
TOTAL COD
BIOLOGICAL SOLIDS
GLYCEROL COD
D.O.
TIME, hrs
TIME, hrs

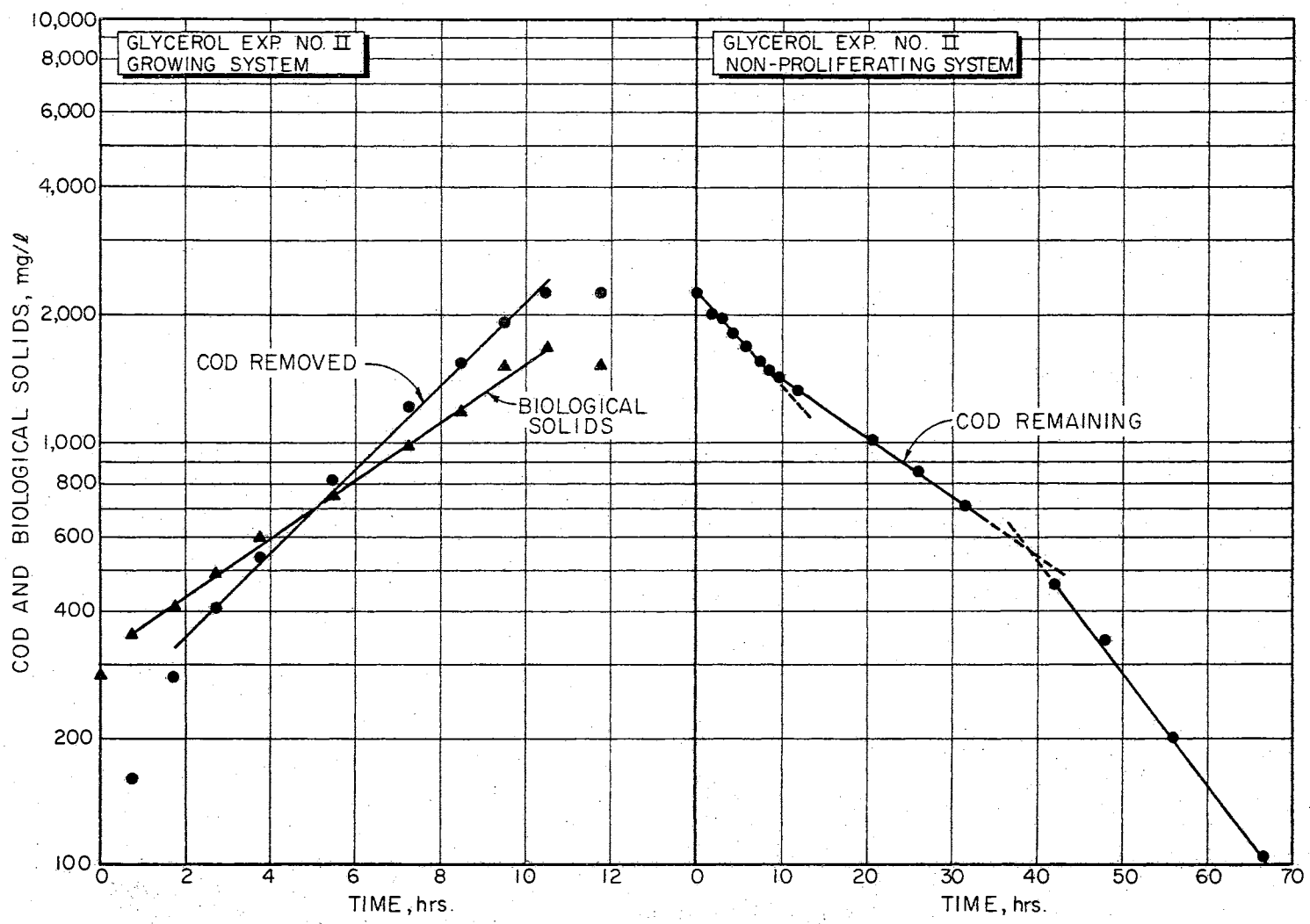


Figure 32 - Semi-log plot of COD removal and solids growth in systems shown in Figure 31.

TABLE XX

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF GLYCEROL(Exp. No. II)

Time Elapsed Hrs:Min	Intermediates and/or Endproducts - COD, mg/l							
	Growing System				Nonproliferating System			
	Total	Acetic Acid	Ribose	Keto Acids	Total	Acetic Acid	Ribose	Keto Acids
00.00	nil	nil	nil	nil	nil	-	-	-
00.45	"	"	trace	"	-	-	-	-
01.45	110	"	"	trace	20	nil	trace	-
02.45	60	"	"	"	60	"	"	-
03.45	nil	"	"	"	nil	"	-	-
05.30	-	"	"	"	385	"	trace	3
07.15	355	"	"	"	305	"	"	3
08.30	270	"	"	2	310	"	"	3
09.30	151	"	"	trace	-	-	-	-
*10.30	88	"	"	"	-	-	-	-
11.45	72	"	"	"	210	nil	trace	5
20.30	65	"	"	"	213	"	"	6
25.45	73	"	2	"	180	"	"	-
31.30	112	"	2	"	118	"	2	6
42.00	81	"	2	"	95	"	-	6
**48.10	104	"	2	"	68	"	2	6
56.00	104	"	2	"	72	"	2	5
***66.30	112	"	2	"	90	"	2	4
72.10	98	"	2	"	80	"	2	3
79.00	90	"	2	"	104	"	3	2
90.30	85	"	2	"	104	"	5	2
96.30	105	"	2	"	104	"	3	trace
103.30	98	"	3	"	105	"	5	"
114.30	81	"	3	"	112	"	5	"
121.30	73	"	4	"	105	"	4	"
128.45	-	"	3		82	"	4	"
139.00	-	"	3	"	-	"	5	"
163.15	-	"	4	"	-	"	7	"
189.00	-	"	4	"	-	"	8	"
216.00	-	"	3	"	108	"	8	"
237.15	53	"	-	"	108	"	-	"
263.15	-	"	3	2	-	"	8	"
287.00	-	"	3	trace	-	"	9	"
309.30	-	"	4	"	124	"	2	"
368.00	-	"	3	"	-	"	-	"
415.30	68	"	3	"	89	"	8	"

* Glycerol eliminated from the medium in growing system

** 23 mg/l propionic acid COD was detected in the medium of nonproliferating system

*** Glycerol eliminated from the medium in the nonproliferating system.

acetic acid was detected in either system. Only traces of ribose and keto acids were present in the growing system. Small amounts of ribose and keto acids persisted in the nonproliferating system. Transient accumulation of a large amount of propionic acid COD (23 mg/l) occurred in the latter system during the late substrate removal phase.

In contrast to the earlier experiment at low initial biological solids, the growing cells in Experiment II preferentially synthesized carbohydrate during the solids accumulation phase. Under nonproliferating conditions, carbohydrate was synthesized only during the initial period of linear increase in solids. Synthesis of protein was considerably lower in the growth system than in the previous experiment. It contributed only 24 per cent to the net increase in solids. A small increase in protein was noted in the nonproliferating system.

Maximum consumption of ammonia nitrogen occurred at the time of the peak in protein synthesis. One hundred twenty-four mg/l of ammonia nitrogen was utilized for growth.

ii) Endogenous Phase

At the time of termination of the experiment (416 hours), 83 per cent of the solids accumulated earlier during growth in the presence of nitrogen was oxidized. The endogenous oxidation of solids was not, however, completed at this time, and further oxidation would have been possible. Very little oxidation of solids occurred during the long period of endogenous metabolism in the nonproliferating system. Only 38 per cent of the solids accumulated during the substrate removal phase was oxidized during 350 hours of endogenous respiration.

It can be seen from the figure that carbohydrate served as the primary endogenous reserve for the growing system. Oxidation of

protein started only after 90 per cent of the accumulated carbohydrate was oxidized. At the time of the termination of the experiment, 90 per cent of the carbohydrate and 64 per cent of the protein synthesized by the cells during the substrate removal phase had been oxidized. It is rather interesting to note that degradation of cellular carbohydrate occurred in the nonproliferating systems even before the exhaustion of the exogenous substrate. Cell protein remained stable throughout the endogenous phase.

Small amounts of ribose and traces of keto acids persisted during the entire endogenous phase of the growing system (Table XX). Somewhat higher quantities of ribose were released by the nonproliferating cells during the endogenous phase.

Thirty-nine per cent of the ammonia nitrogen consumed earlier by the cells for growth was released into the medium during the endogenous metabolism in the growing system. No organic nitrogen was released. Neither form of nitrogen was released by the nonproliferating cells.

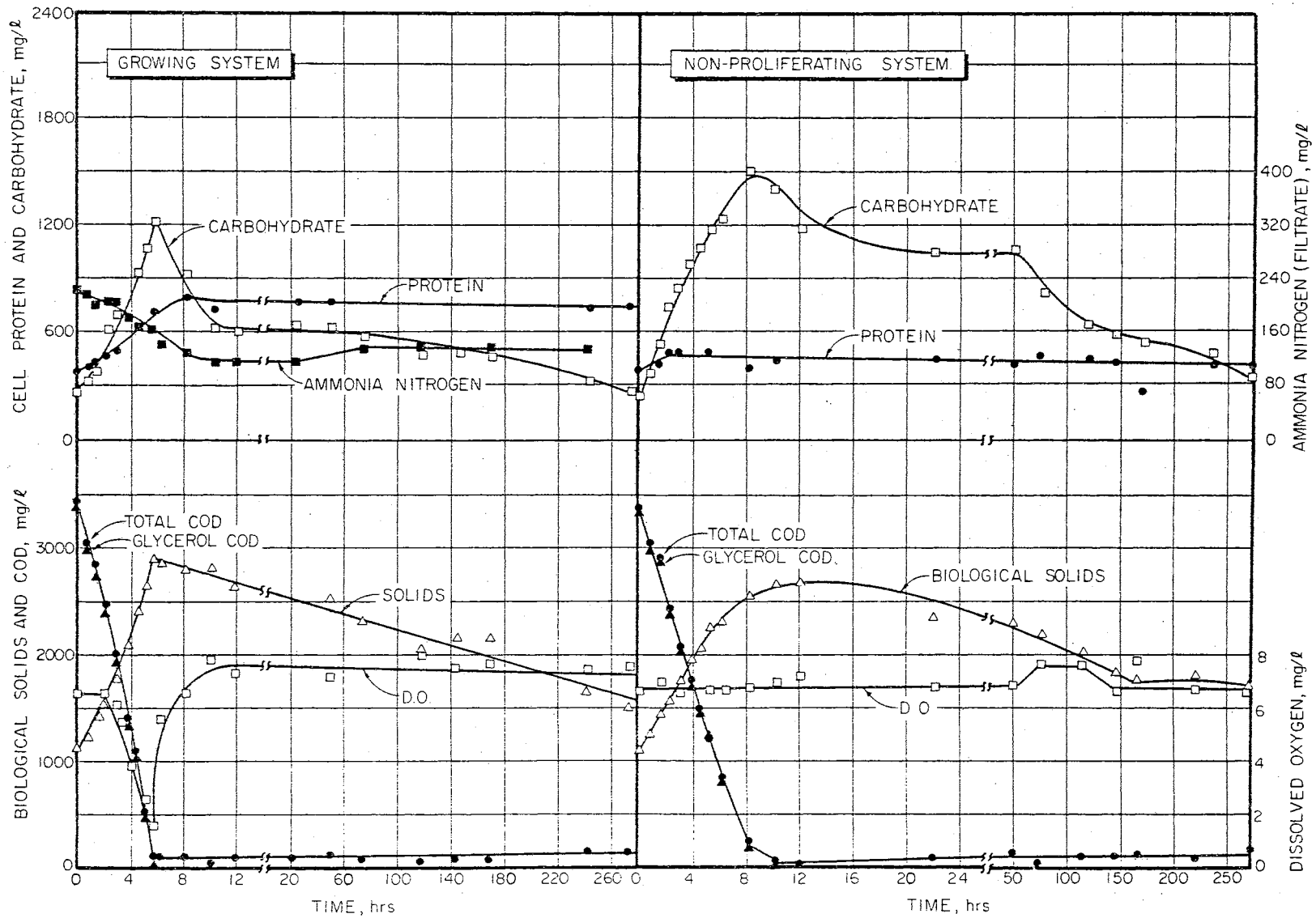
c. Experiment III. Metabolism of Glycerol by Growing and Nonproliferating Cells at Low Initial COD:Biological Solids Ratio

Figure 33 shows the solids accumulation and endogenous phases of the systems with an initial COD:biological solids ratio of 3.

i) Solids Accumulation Phase

The difference in the rate of substrate removal under growing and nonproliferating conditions was much less pronounced than it was when higher COD:solids ratios were employed (compare Figures 29, 31, and 33). COD removal appeared to approach zero order kinetics in both systems. Solids increase was exponential in the growing system (Figure 34), whereas it was linear during the major part of the

Figure 33 - Comparison of solids accumulation and endogenous phases under growing and nonproliferating conditions during metabolism of glycerol at low initial COD:biological solids ratio (Experiment III).


GROWING SYSTEM
NON-PROLIFERATING SYSTEM
CELL PROTEIN AND CARBOHYDRATE, mg/ℓ
AMMONIA NITROGEN (FILTRATE), mg/ℓ
BIOLOGICAL SOLIDS AND COD, mg/ℓ
DISSOLVED OXYGEN, mg/ℓ
CARBOHYDRATE
PROTEIN
AMMONIA NITROGEN
TOTAL COD
GLYCEROL COD
SOLIDS
BIOLOGICAL SOLIDS
D.O
TIME, hrs
TIME, hrs

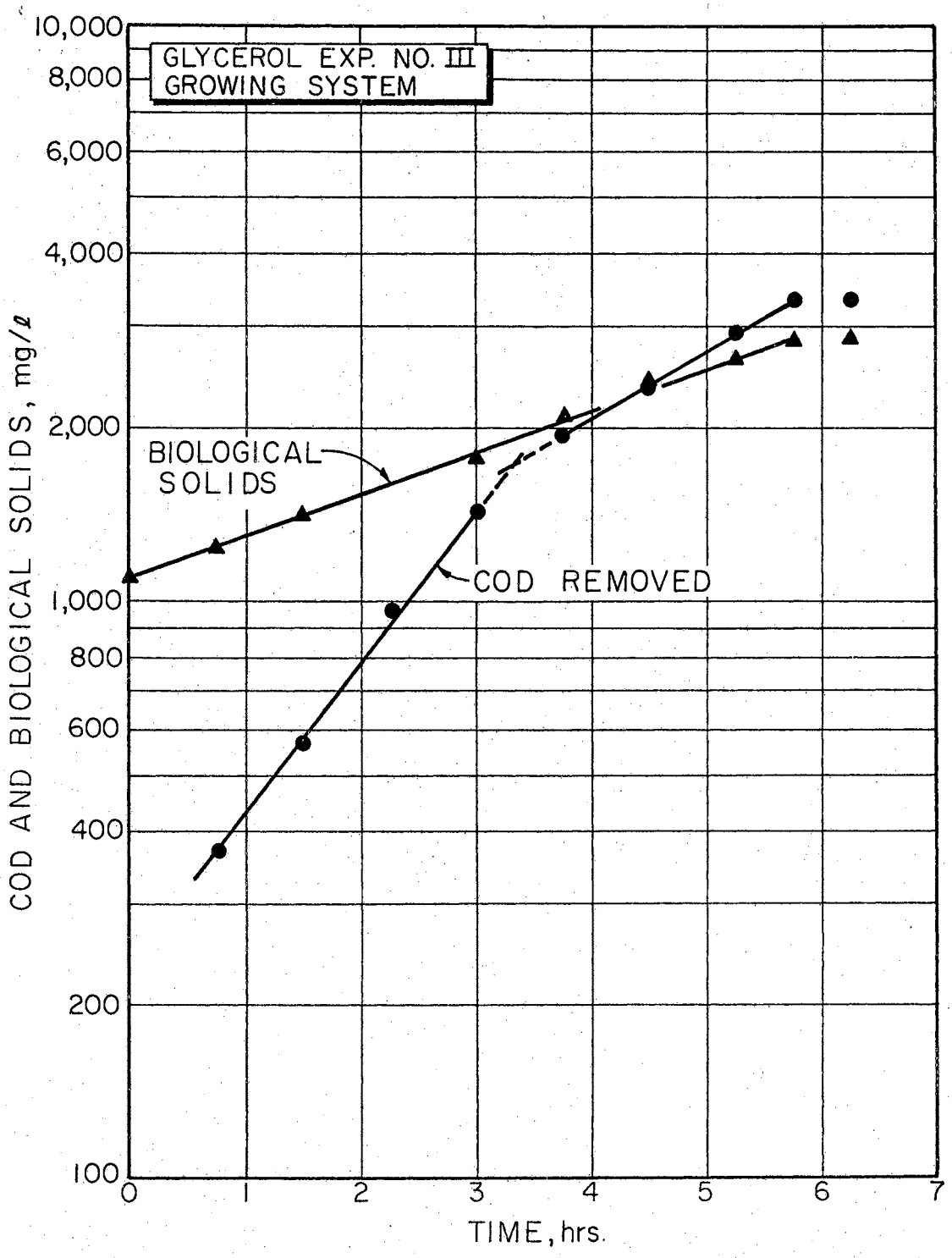


Figure 34 - Semi-log plot of COD removal and solids growth in growing system shown in Figure 33.

substrate removal phase in the nonproliferating system. There was essentially no difference in cell yield between the two systems.

Table XXI shows the extracellular metabolic intermediates and/or endproducts secreted by the cells under both conditions. There was no difference in the amounts of total intermediates released in both systems during the solids accumulation phase. Much smaller amounts of metabolic intermediates accumulated in these systems than in experiments conducted with lower initial solids (Compare Tables XIX and XXI). No acetic acid could be detected in either system. Only traces of ribose and keto acids were detected in both systems.

Carbohydrate synthesis was considerably enhanced in the nonproliferating system. As much as 76 per cent of the solids accumulation in these cells was due to the synthesis of carbohydrate. These cells had a very high content (53 per cent) of carbohydrate at the time of maximum solids production. Protein content was only 17 per cent.

ii) Endogenous Phase

Endogenous oxidation of solids was not completed in the growing system at the time of termination of the experiment (273 hours) when 77 per cent of the accumulated solids had been oxidized. Endogenous metabolism had been completed in the nonproliferating system; only 58 per cent of the accumulated solids was oxidized in this system. However, this degree of oxidation was much greater than that observed when lower initial biological solids were employed (compare Figures 29 and 33).

It is interesting to note that almost total oxidation of carbohydrate synthesized under balanced growth conditions occurred during the endogenous phase. As much as 89 per cent of the accumulated carbohydrate was utilized by the nonproliferating cells during the corresponding

TABLE XXI

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF GLYCEROL (Exp. No. III)

Time Elapsed Hrs:Min	Intermediates and/or Endproducts - COD, mg/l							
	Growing System				Nonproliferating System			
	Total	Acetic Acid	Ribose	Keto Acids	Total	Acetic Acid	Ribose	Keto Acids
00.00	10	nil	trace	nil	25	nil	nil	-
00.45	70	"	"	trace	75	"	"	-
01.30	-	"	"	"	nil	"	"	-
02.15	25	"	"	-	65	"	"	trace
03.00	50	"	"	-	nil	"	trace	"
03.45	60	"	"	-	50	"	"	"
04.30	25	"	"	-	40	"	"	-
05.15	33	"	"	-	nil	"	"	-
*05.45	104	"	"	2	-	"	"	-
06.15	99	"	"	-	10	"	"	2
08.15	94	"	"	trace	32	"	"	2
**10.15	45	"	"	2	74	"	"	-
12.00	79	"	"	2	59	"	"	-
22.15	74	"	"	-	104	"	"	2
50.00	133	"	2	2	163	"	"	2
74.15	69	"	2	2	59	"	2	2
118.10	50	"	3	-	118	"	3	2
145.55	80	"	2	trace	103	"	4	trace
169.55	71	"	2	-	150	"	7	2
242.40	150	"	4	-	87	"	3	2
273.10	142	"	3	3	197	"	6	trace

* Glycerol eliminated from the medium in growing system
** Glycerol eliminated from the medium in nonproliferating system.

period of endogenous metabolism. Cellular protein seemed to be stable in both systems during the endogenous oxidation phase observed during the study.

Only 21 per cent of the total ammonia nitrogen consumed by the cells during the solids accumulation phase was released into the medium of the growing system during the endogenous phase. There was no evidence of organic nitrogen accumulation in the system. Neither ammonia nor organic nitrogen could be detected in the nonproliferating system throughout the course of the experiment. The COD contributed by the soluble endproducts was somewhat higher in the nonproliferating system (Table XXI). Ribose and keto acids were released by the cells in both systems.

2. Metabolism of Sorbitol

Long-term batch studies were conducted with this substrate at three different initial COD:biological solids ratios. Table XXII shows the experimental outline for these studies.

TABLE XXII

EXPERIMENTAL OUTLINE FOR LONG-TERM BATCH EXPERIMENTS ON SORBITOL

	Growing System				Nonproliferating System			
Exp. No.	Initial COD mg/l	Initial Biolog. Solids mg/l	Initial COD / Initial Biolog. Solids	Duration hrs	Initial COD mg/l	Initial Biolog. Solids mg/l	Initial COD / Initial Biolog. Solids	Duration hrs
I	2290	88	26.0	944	2320	93	25.0	944
II	2300	163	14.1	1218	2300	173	13.3	1050
III	2240	775	2.9	720	2210	775	2.9	720

a. Experiment I. Metabolism of Sorbitol by Growing and Nonproliferating Cells at High Initial COD:Biological Solids Ratio

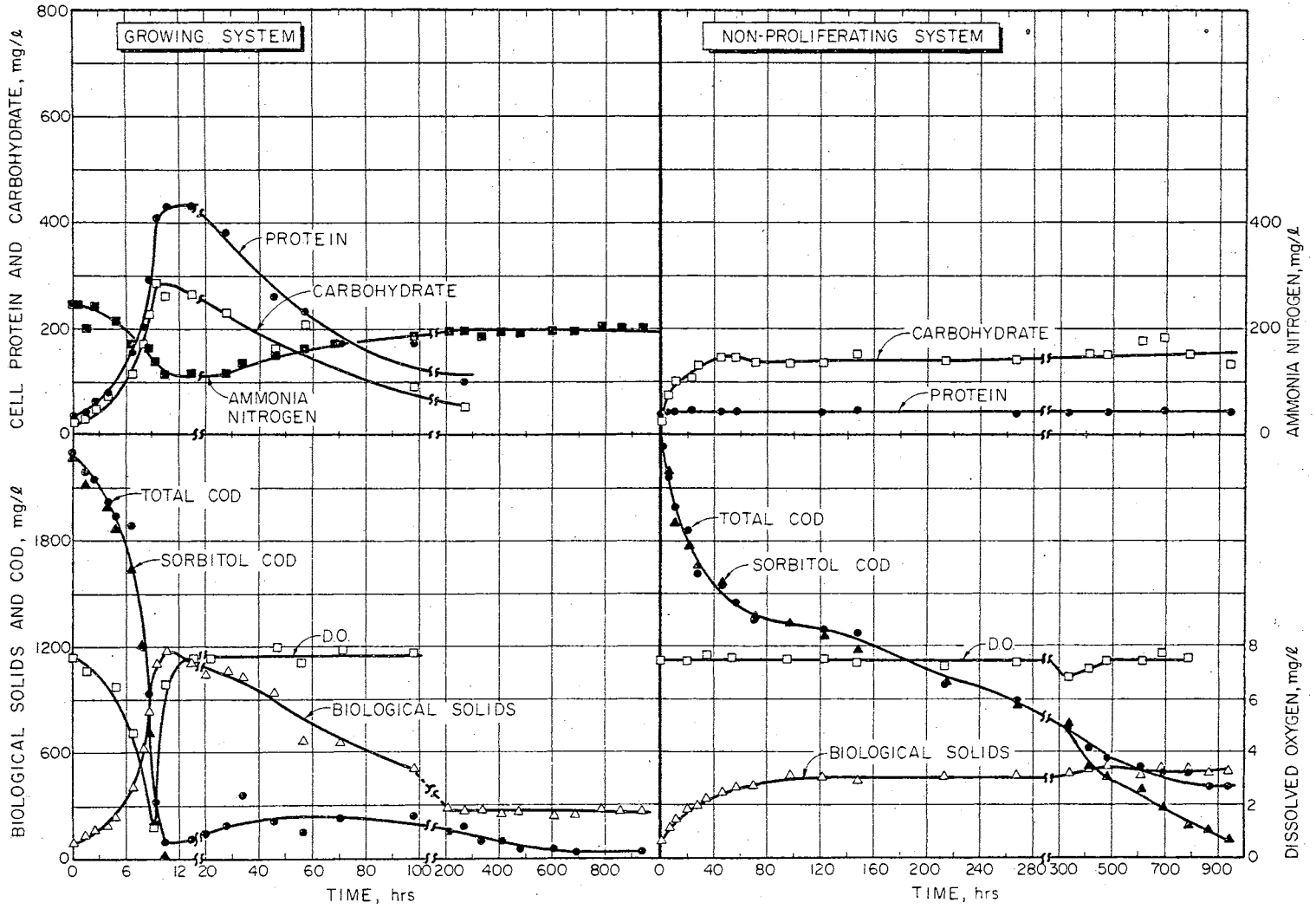
Figure 35 shows the response when an initial COD:solids ratio of 26 was employed.

i) Solids Accumulation Phase

Substrate removal and solids growth were exponential in the growing system (Figure 36). However, under nonproliferating conditions, COD removal showed three phases of decreasing first-order kinetics (Figure 36) during the first 694 hours (about 29 days). The reaction rate was reduced in the succeeding phases. There was a lag of 70 hours in this system after the initial phase of COD removal. At the termination of the experiment (944 hours), 17 per cent of the initial COD remained unutilized by the cells.

Cell yield was considerably lower in the nonproliferating system. Accumulation of solids occurred only during the initial phase of COD removal. Table XXIII shows the amounts of various metabolic intermediates and/or endproducts present in the systems during the course of the experiment. Small amounts of fructose (2 to 4 mg/l), keto acids (2 to 3 mg/l), and ribose (2 to 12 mg/l) appeared in both systems during the solids accumulation phase. Propionic acid was detected in the filtrate of one sample (see footnote of Table XXIII). Acetic acid appeared transiently in the growing system during the final hours of growth. Small amounts (5 to 6 mg/l) were present in the nonproliferating system during the early phase of COD removal. However, large amounts of metabolic intermediates accumulated in this system during the last stage of substrate removal. Significant amounts of ribose accumulated in the system during this period. Sorbitol removal was not

Figure 35 - Comparison of solids accumulation and endogenous phases during metabolism of sorbitol under growing and nonproliferating conditions at high initial COD:biological solids ratio (Experiment I).


GROWING SYSTEM
NON-PROLIFERATING SYSTEM
CELL PROTEIN AND CARBOHYDRATE, mg/ℓ
AMMONIA NITROGEN, mg/ℓ
BIOLOGICAL SOLIDS AND COD, mg/ℓ
DISSOLVED OXYGEN, mg/ℓ
PROTEIN
CARBOHYDRATE
AMMONIA NITROGEN
TOTAL COD
SORBITOL COD
D.O.
BIOLOGICAL SOLIDS
TIME, hrs
TIME, hrs

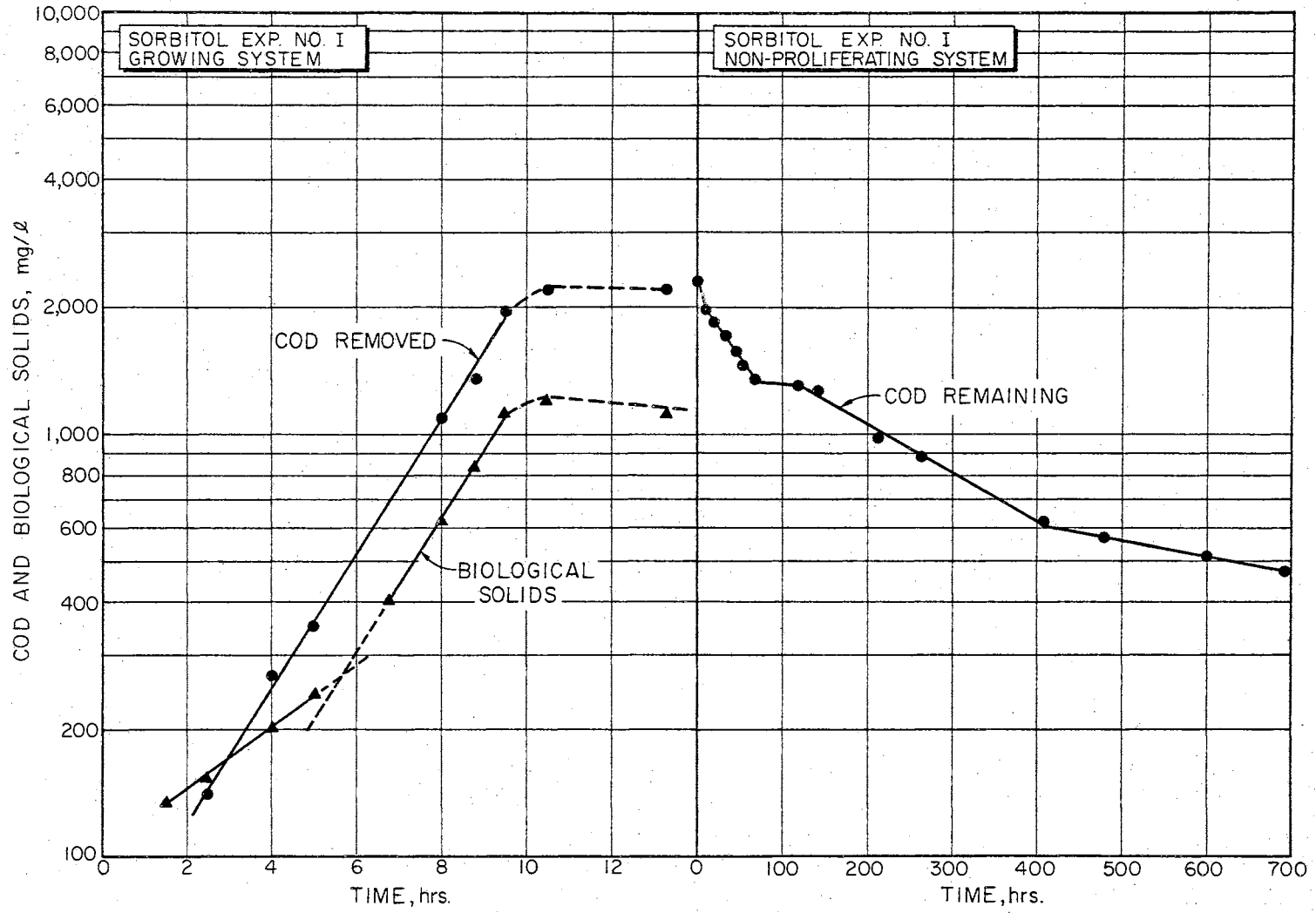


Figure 36 - Semi-log plot of COD removal and solids growth in systems shown in Figure 35.

TABLE XXIII

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF SORBITOL (Exp. No. I)

Time Elapsed Hrs:Min	Intermediates and/or Endproducts - COD, mg/l									
	Growing System					Nonproliferating System				
	Total	Acetic Acid	Ribose	Fructose	Keto Acids	Total	Acetic Acid	Ribose	Fructose	Keto Acids
00.00	nil	nil	nil	nil	nil	nil	-	-	-	-
00.30	"	"	"	"	"	"	-	-	-	-
01.30	80	"	trace	trace	trace	"	nil	trace	-	trace
02.30	-	"	"	3	-	"	"	-	-	-
04.00	40	4	"	trace	trace	"	"	-	-	-
*05.00	75	6	2	"	"	"	"	-	-	-
06.45	-	5	trace	"	"	"	"	-	-	-
08.00	nil	14	"	"	"	"	"	-	-	-
09.30	97	38	2	4	2	"	"	-	-	-
**10.30	67	nil	trace	nil	trace	90	"	trace	-	trace
13.30	107	"	"	"	"	168	"	"	-	"
19.50	143	"	"	"	"	95	6.0	"	-	"
28.10	184	"	2	"	"	nil	5.0	-	-	-
34.00	-	"	3	"	"	-	-	-	-	-
46.00	215	"	3	"	"	nil	-	-	-	-
57.00	132	"	4	"	"	"	-	-	-	-
70.30	230	"	4	"	"	"	-	-	-	-
98.00	248	"	5	"	"	-	-	-	-	-
122.00	-	-	-	-	-	30	nil	2	-	-
148.30	248	nil	6	nil	trace	90	"	-	-	-
213.30	156	"	4	"	"	nil	"	6	-	-
267.30	185	"	4	"	"	8	"	5	-	-
335.00	87	"	trace	"	"	nil	-	-	-	-
409.45	93	"	"	"	"	100	nil	6	-	2
482.05	49	"	2.0	"	"	110	"	8	2	2
604.00	59	"	trace	"	"	128	"	8	2	2
693.45	35	"	"	"	"	178	"	8	3	3
790.00	-	-	-	-	-	289	"	11	3	2
862.00	-	-	-	-	-	236	"	6	2	2
***944.00	36	nil	trace	trace	trace	282	"	12	3	trace

*14 mg/l propionic acid COD was detected in the growing system
**Sorbitol eliminated from medium in growing system
***108 mg/l sorbitol COD still present in medium in nonproliferating system.

complete in this system at the time of termination of the experiment. Seventy-two per cent of the COD remaining in the system at this time was compounds other than sorbitol.

Synthesis of protein was higher than that of carbohydrate in the growing system. Carbohydrates constituted only 22 per cent of the net solids synthesis. The cells contained 22 per cent carbohydrate and 36 per cent protein at the time of maximum solids accumulation (10 hours and 30 minutes). Synthesis of carbohydrate occurred only during the first stage of COD removal in the nonproliferating system, and accounted for 32 per cent of accumulated solids. No net increase in protein was observed.

Maximum removal of ammonia nitrogen (128 mg/l) occurred at the time of maximum solids accumulation in the growing system, which corresponded to the peak in protein synthesis.

ii) Endogenous Phase

Eighty-three per cent of the total biological solids synthesized by the growing system was oxidized during the first 260 hours of the endogenous phase. Prolonging the aeration for another 580 hours beyond this stage did not result in further oxidation of solids. Eighty-four per cent of protein and 89 per cent of carbohydrate synthesized by the cells during the solids accumulation phase was oxidized during the endogenous phase. At the end of the endogenous phase, the cells contained 37 per cent protein and 18 per cent carbohydrate, which was not unlike the initial composition.

Release of ammonia nitrogen by the cells in the growing system began at the onset of the endogenous phase and reached its maximum level at the termination of this phase, i.e., NH_3 was not released

beyond the period of decreasing solids concentration. No organic nitrogen was present in the medium at any time during the endogenous phase. Small amounts of ribose (2 to 6 mg/l) persisted in the medium throughout the major portion of the endogenous phase.

The nonproliferating system did not enter the endogenous phase during the period of the experiment, i.e., biological solids did not decrease after attaining an upper level; some original exogenous substrate was present throughout the experiment.

b. Experiment II. Metabolism of Sorbitol by Growing and Nonproliferating Cells at an Intermediate Ratio of Initial COD:Biological Solids

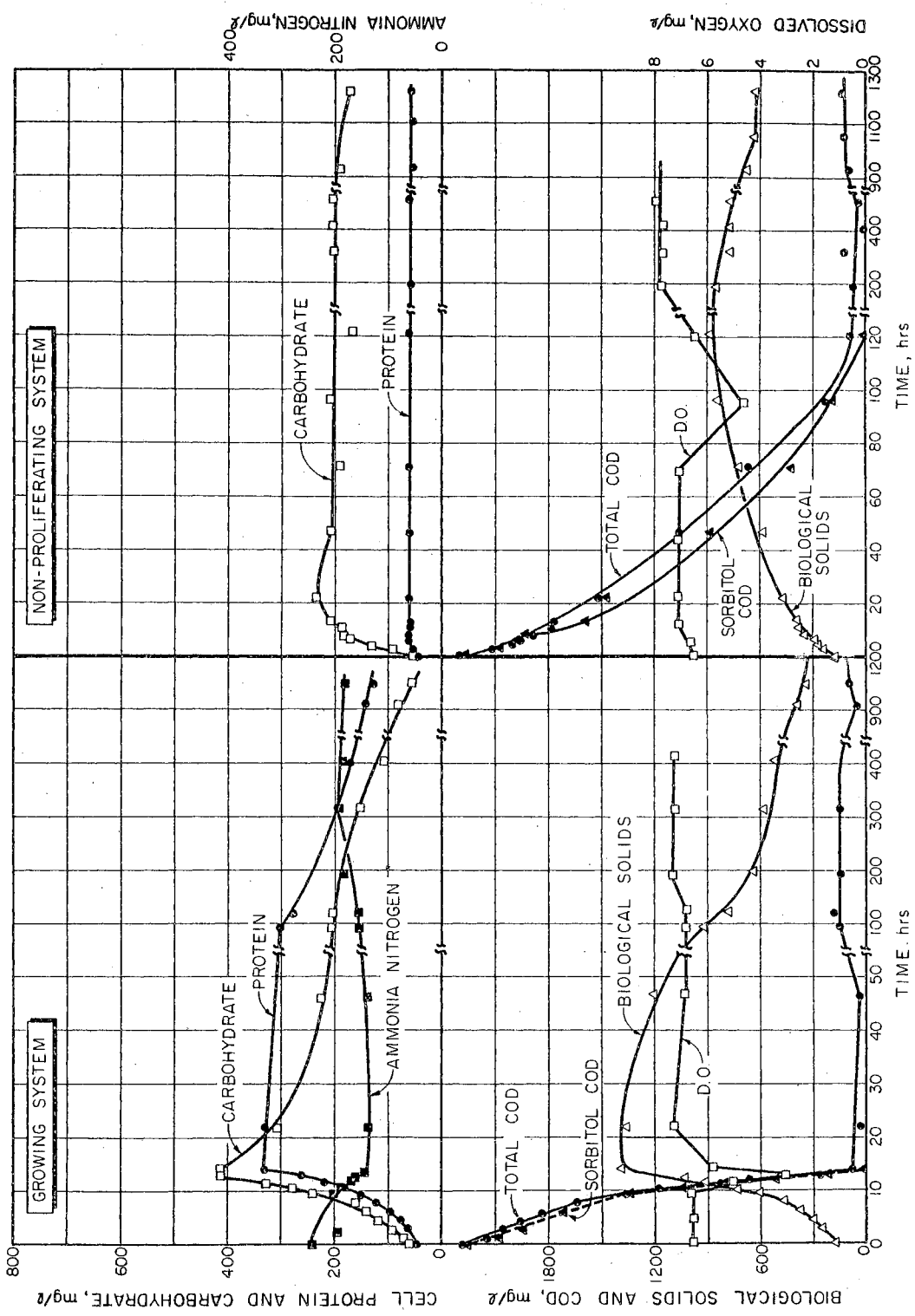
Figure 37 shows the response when the initial COD:biological solids ratio was 14 in the growing system, and 13 in the nonproliferating system.

i) Solids Accumulation Phase

COD removal and solids growth occurred logarithmically in the growing system (Figure 38). COD removal rate was considerably increased in the nonproliferating system compared to the similar system in the previous experiment. A semi-log plot of the COD remaining in the system versus time showed two phases of first-order removals (Figure 38). Ninety-six per cent of the initial COD was removed within the first 120 hours. In the growing system, however, comparable COD removal was achieved in about one-tenth of this time. Cell yield in the absence of nitrogen was somewhat higher than observed in the previous experiment. It was, however, considerably lower (by 24 per cent) than in the corresponding growing system.

Table XXIV shows the amounts and types of metabolic intermediates and endproducts released by the cells. Small amounts of acetic acid

Figure 37 - Comparison of solids accumulation and endogenous phases during metabolism of sorbitol under growing and nonproliferating conditions at an intermediate ratio of initial COD:biological solids (Experiment II).


GROWING SYSTEM
NON-PROLIFERATING SYSTEM
CELL PROTEIN AND CARBOHYDRATE, mg/ℓ
BIOLOGICAL SOLIDS AND COD, mg/ℓ
AMMONIA NITROGEN, mg/ℓ
DISSOLVED OXYGEN, mg/ℓ
CARBOHYDRATE
PROTEIN
AMMONIA NITROGEN
TOTAL COD
SORBITOL COD
BIOLOGICAL SOLIDS
D.O
TIME, hrs

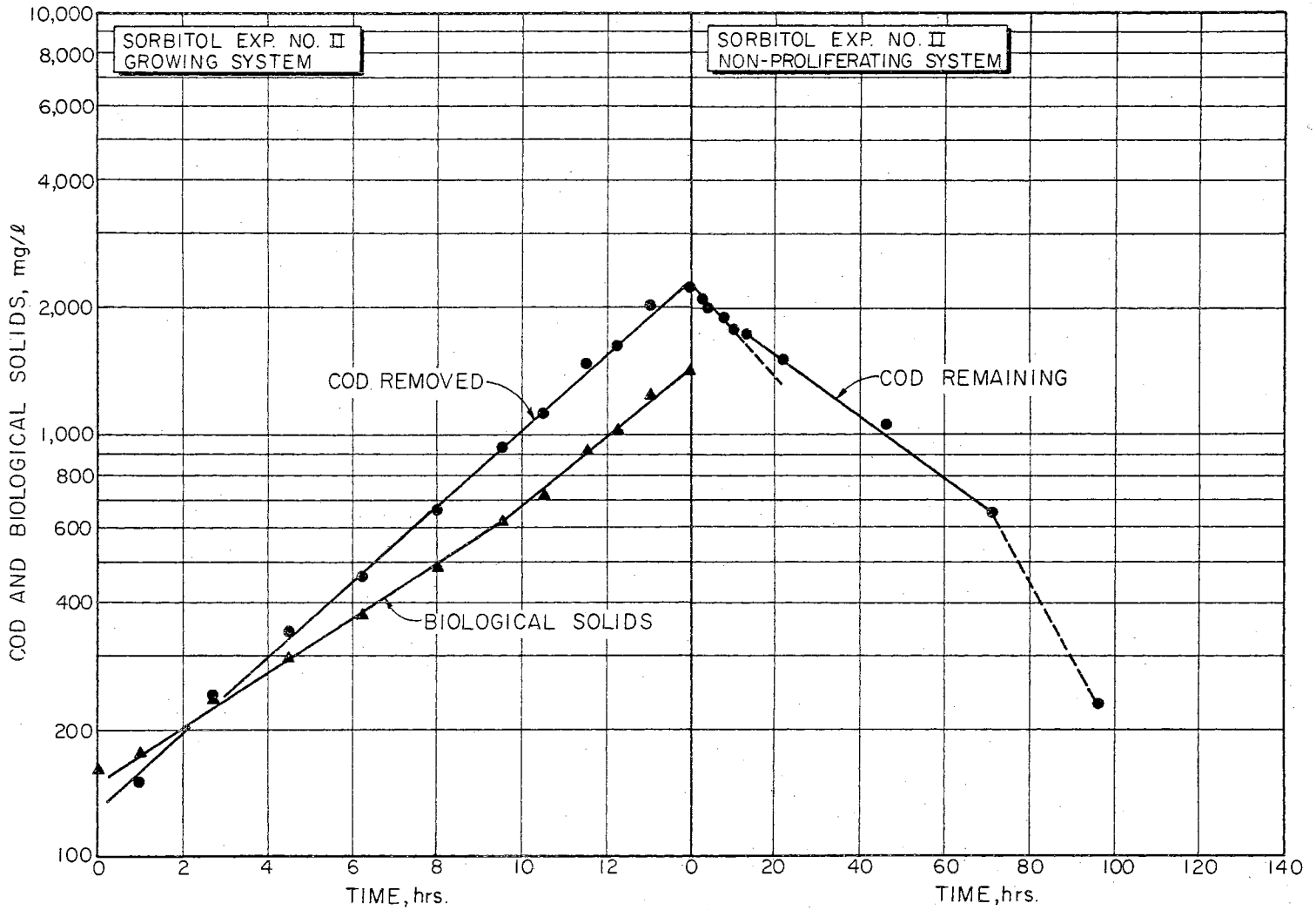


Figure 38 - Semi-log plot of COD removal and solids growth in systems shown in Figure 37.

TABLE XXIV

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF SORBITOL (Exp. No. II)

Time Elapsed Hrs:Min	Intermediates and/or Endproducts - COD, mg/l									
	Growing System					Nonproliferating System				
	Total	Acetic Acid	Ribose	Fructose	Keto Acids	Total	Acetic Acid	Ribose	Fructose	Keto Acids
00.00	-	-	-	-	-	-	-	-	-	-
01.00	60	trace	trace	4	trace	-	-	-	-	-
02.45	120	6	"	4	"	30	nil	trace	-	trace
04.30	-	4	"	4	"	nil	-	-	-	-
06.15	130	11	"	3	"	"	-	-	-	-
08.00	-	-	-	3	"	"	-	-	-	-
9.30	nil	-	-	-	-	-	-	-	-	-
10.30	122	5	trace	3	trace	-	-	-	3	-
11.30	15		3	-	"	-	-	-	-	-
12.15	137	8	2	3	"	-	-	-	-	-
13.00	43	8	4	3	"	165	nil	trace	3	-
*13.50	86	nil	trace	-	"	-	-	-	-	-
22.15	32	"	"	nil	"	45	nil	trace	3	trace
46.35	46	"	"	-	"	165	"	"	3	"
70.55	142	"	"	-	"	237	"	"	trace	"
96.00	157	"	3	-	"	35	"	2	3	"
**120.30	172	"	4	-	"	81	"	trace	trace	"
197.30	148	"	3	-	"	64	"	"	"	"
317.30	148	"	3	-	"	114	"	2	"	"
407.00	-	"	-	-	"	nil	"	2	"	"
503.30	24	"	trace	-	"	32	"	3	"	"
575.30	41	"	"	-	"	49	"	trace	"	"
655.15	25	"	"	-	"	53	"	2	-	"
930.00	53	"	"	-	"	98	"	5	-	"
1050.00	101	"	"	-	"	121	"	3	-	"
1218.00	-	"	"	-	"	138	"	2	-	"

*Sorbitol eliminated from medium in growing system
**Sorbitol eliminated from medium in nonproliferating system.

and ribose were released by the cells in the growing system. Fructose was present in the medium of both systems during the metabolism of sorbitol.

In contrast with the previous experiment, the growing cells of the present experiment synthesized smaller amounts of protein and larger amounts of carbohydrate. Carbohydrate synthesis accounted for 30 and 22 per cent of the solids synthesized by the growing system, and the nonproliferating system, respectively. Cell carbohydrate and protein peaked at the time of maximum solids accumulation in the growing system, and constituted 30 per cent and 24 per cent, respectively, of the cell dry weight. At the time of substrate removal, carbohydrate amounted to 23 per cent of the dry weight of the cells in the nonproliferating system. Ninety-seven mg/l of ammonia nitrogen was utilized by the growing cells during the solids accumulation phase.

ii) Endogenous Phase

Endogenous oxidation of biological solids was not completed at the time of termination of the experiment (1050 hours) in the growing system. However, by this time, 85 per cent of the net solids synthesized during the solids accumulation phase had been oxidized. Under nonproliferating conditions the endogenous phase had been completed at the time of termination of the experiment (1218 hours). Only 37 per cent of the solids accumulated in the absence of nitrogen was oxidized during the endogenous phase.

The extent of endogenous utilization of protein and carbohydrate varied greatly in the two systems. Almost 100 per cent of the carbohydrate and 72 per cent of the protein synthesized in the presence of nitrogen were oxidized during the endogenous phase. However, only 34

percent of the carbohydrate accumulated by the nonproliferating cells was oxidized.

It can be seen from Figure 37 that very little protein was utilized by the cells in the growing system during the first 100 hours of the endogenous phase. About 60 per cent of the carbohydrate accumulated by the cells had been oxidized before the oxidation of protein began. Cell protein remained stable throughout the endogenous phase of the nonproliferating system.

Release of ammonia nitrogen by the cells occurred during the oxidation of protein in the growing system. Fifty per cent of the ammonia nitrogen utilized for growth was returned to the medium during the prolonged endogenous metabolism of the cells. Compounds containing organic nitrogen were absent in both systems during the entire duration of the experiments. Ribose was detected in the medium of the growing systems during the early endogenous phase. Small amounts of ribose were present in the nonproliferating system throughout the endogenous phase (Table XXIV).

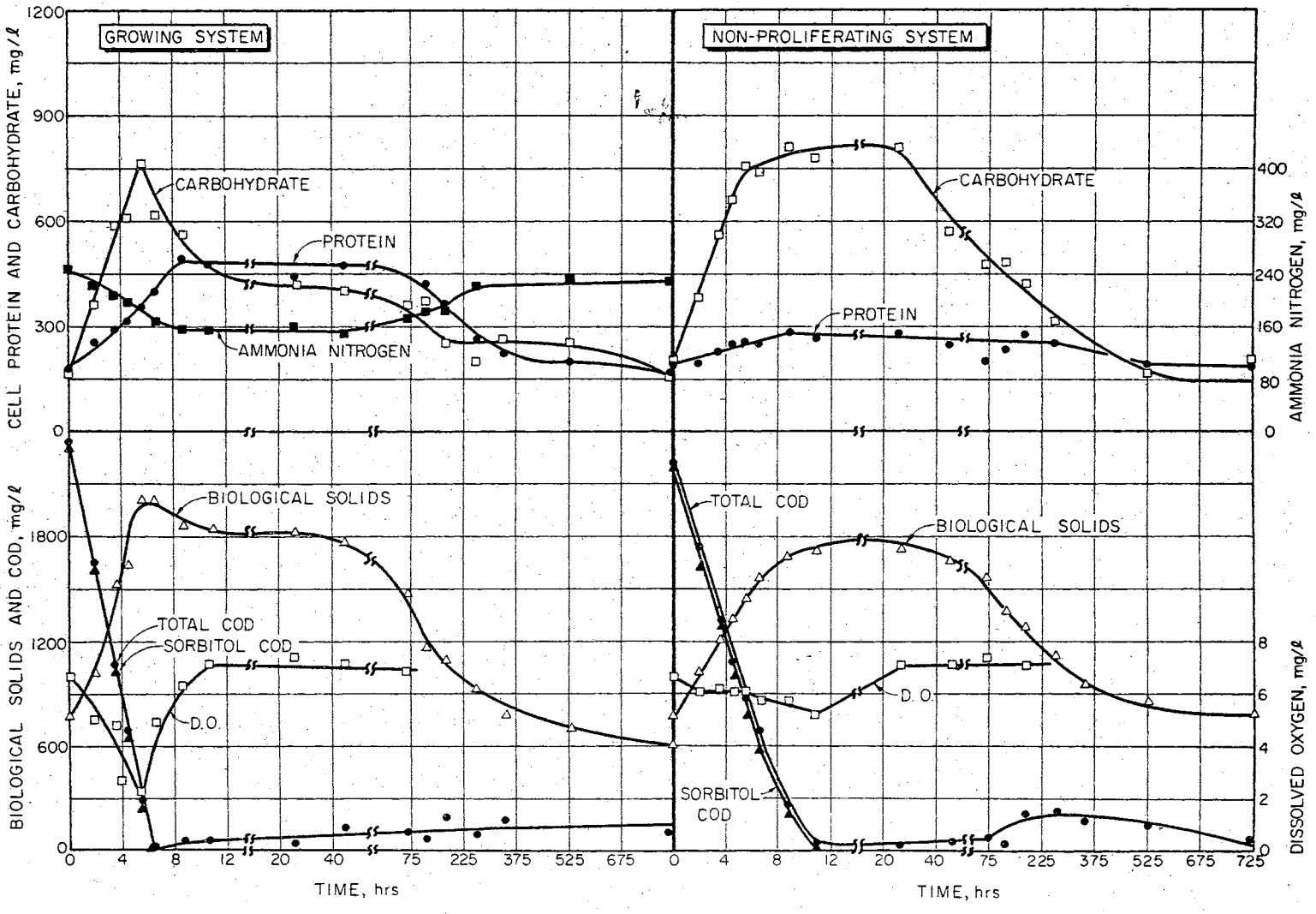
c. Experiment III. Metabolism of Sorbitol by Growing and Nonproliferating Cells at Low Initial COD:Biological Solids Ratio

Figure 39 shows the changes in the parameters measured in the systems with an initial COD:solids ratio of 3.

i) Solids Accumulation Phase

COD removal followed zero-order kinetics in both systems, although the rate was somewhat higher in the growing system. Ninety-eight per cent COD removal was obtained in the nonproliferating system at the end of 11 hours of aeration. Solids growth was exponential in the growing system (Figure 40), and linear in the nonproliferating system.

Figure 39 - Comparison of solids accumulation and endogenous phases during metabolism of sorbitol under growing and nonproliferating conditions at low initial COD:biological solids ratio (Experiment III).


GROWING SYSTEM
NON-PROLIFERATING SYSTEM
CELL PROTEIN AND CARBOHYDRATE, mg/ℓ
AMMONIA NITROGEN, mg/ℓ
BIOLOGICAL SOLIDS AND COD, mg/ℓ
DISSOLVED OXYGEN, mg/ℓ
CARBOHYDRATE
PROTEIN
AMMONIA NITROGEN
BIOLOGICAL SOLIDS
TOTAL COD
SORBITOL COD
D.O.
TIME, hrs
TIME, hrs

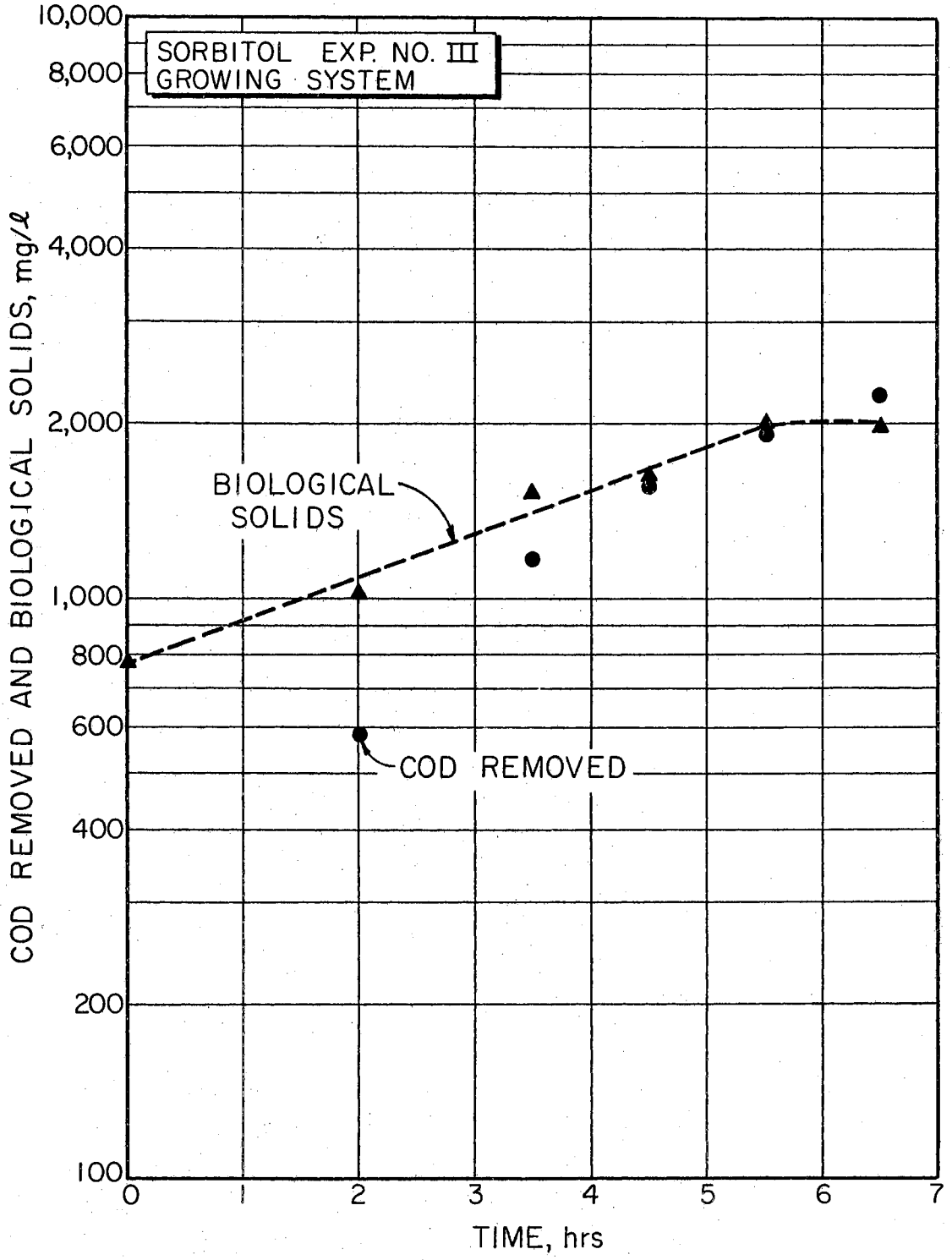


Figure 40 - Semi-log plot of COD removal and solids growth in growing system shown in Figure 39.

The wide difference in cell yield between the two systems noted in earlier experiments was considerably reduced in this experiment.

A very small amount of metabolic intermediates was released by either system during the metabolism of sorbitol (Table XXV). Small amounts of fructose and keto acids were produced by the cells in both systems.

Carbohydrate synthesis was considerably enhanced in the present experiment involving higher initial biological solids. It was the predominant synthetic product in the solids accumulation phase of both systems. It accounted for 37 per cent of the net increase in solids in the growing system, and 62 per cent in the nonproliferating system. Maximum accumulation of carbohydrate occurred at the time of the peak in solids synthesis in both systems, and amounted to 47 per cent of the dry weight of the cells in the nonproliferating system and 38 per cent in the growing system. More than 40 per cent of the protein synthesized by the growing system was produced during the early endogenous phase at the expense of the cellular carbohydrate. Eighty-nine mg/l of ammonia nitrogen had been utilized at the time of maximum synthesis of protein. A small increase in the protein content of the nonproliferating system was noted.

ii) Endogenous Phase

The cells in the growing system had not completed the endogenous phase at the time of termination of the experiment (720 hours); i.e., solids were still decreasing. The system had synthesized 1217 mg/l of biological solids during the growth phase. During the 713 hours of endogenous metabolism, 1397 mg/l of solids were oxidized. Total solids accumulated by the nonproliferating cells amounted to 950 mg/l, and

TABLE XXV

RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING METABOLISM OF SORBITOL (Exp. No. III)

Time Elapsed Hrs:Min	Intermediates and/or Endproducts - COD, mg/l									
	Growing System					Nonproliferating System				
	Total	Acetic Acid	Ribose	Fructose	Keto Acids	Total	Acetic Acid	Ribose	Fructose	Keto Acids
00.00	-	-	-	-	-	-	-	-	-	-
02.00	43	nil	-	3	trace	113	nil	trace	3	4
03.30	25	"	trace	3	"	18	"	"	-	-
04.30	25	"	2	2	2.0	100	"	"	3	2.0
05.30	38	"	-	2	2.0	110	"	2.0	3	2.0
*06.30	nil	"	trace	nil	-	105	"	trace	3	trace
08.45	50	"	"	"	trace	62	"	"	-	-
**10.45	50	"	"	"	2.0	55	"	"	trace	trace
26.15	35	"	"	"	2.0	20	"	2.0	nil	-
45.10	138	"	"	"	trace	50	"	trace	"	trace
74.15	94	"	2.0	"	3.0	84	"	4.0	"	"
117.10	69	"	3	"	-	30	"	4.0	"	"
170.15	203	"	3	"	trace	203	"	4.0	"	"
262.15	98	"	3	"	2.0	224	"	8.0	"	"
342.45	182	"	2.0	"	trace	163	"	3.0	"	"
527.45	-	"	trace	"	2.0	152	"	trace	"	"
719.45	92	"	"	"	2.0	61	"	"	"	2.0

*Sorbitol eliminated from medium in growing system
**Sorbitol eliminated from medium in nonproliferating system.

930 mg/l of solids were destroyed during the endogenous phase. This system appeared to reach the end of the endogenous metabolism during the last stage of the experiment.

All of the protein and carbohydrate synthesized by the growing system was oxidized during endogenous metabolism. Similarly, almost total oxidation of accumulated carbohydrate was noted in the nonproliferating system. It is doubtful whether any further oxidation of carbohydrate could take place in this system, since the solids curve tended to become asymptotic to a lower limit.

Protein resisted oxidation during the initial stage of endogenous metabolism in the growing system. Ammonia was released by the cells during the period when protein was oxidized. As much as 80 per cent of the ammonia nitrogen utilized during growth was released into the medium during the endogenous phase. No change in the protein content of the nonproliferating system was observed. Organic nitrogen-containing compounds were absent in both systems. Significant amounts of ribose were present in the nonproliferating system.

CHAPTER VI

DISCUSSION

A. Continuous Flow Experiments

1. Effect of Dissolved Oxygen on Steady State Behavior

The average steady state values of various parameters prior to an increase in the feed concentration, after the transient response to the increased feed and after a return to the initial feeding level, as well as the average DO levels prevailing in these steady states were shown in Table III. In order to facilitate comparison of values of all of the steady state parameters measured at different DO levels, the results summarized in Table III are plotted in Figures 41 and 42.

Figure 42G shows that COD removal efficiency ranged from 88 to 99 per cent, and the average steady state values of 93 per cent COD removal were recorded at DO levels of 7.4, 3.2, and 0.4 mg/l. Thus it would appear that the efficiency of the system under steady state operation was independent of dissolved oxygen concentration over the range studied. Similarly, glucose removals which varied from 96 to 100 per cent did not show any dependency on the prevailing DO levels.

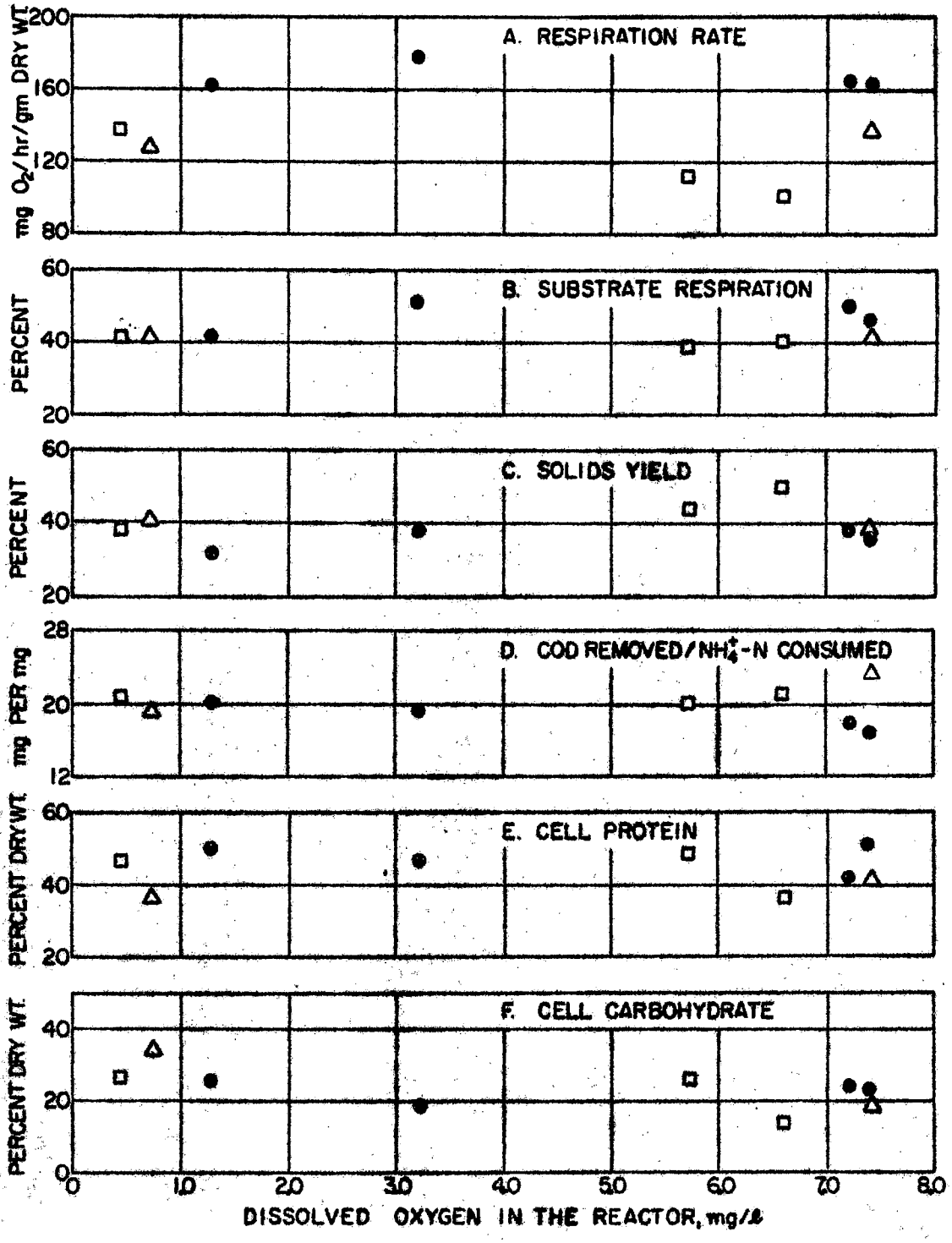
Figure 42I shows the average percent of influent COD converted into metabolic intermediates and endproducts. It ranged from 2 at a DO level of 6.6 mg/l (Experiment I) to 8 at 0.7 mg/l (Experiment IV). This difference could not be attributed to the effect of DO tension,

Figure 41 - Effect of dissolved oxygen concentration on various parameters during "steady state" operation

● before application of shock load

□ after application of shock load

△ after withdrawal of shock load


A. RESPIRATION RATE
mg O_2/hr/gm DRY WT.
200
160
120
80
B. SUBSTRATE RESPIRATION
PERCENT
60
40
20
C. SOLIDS YIELD
PERCENT
60
40
20
D. COD REMOVED/NH_4^+-N CONSUMED
mg PER mg
28
20
12
E. CELL PROTEIN
PERCENT DRY WT.
60
40
20
F. CELL CARBOHYDRATE
PERCENT DRY WT.
40
20
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
DISSOLVED OXYGEN IN THE REACTOR, mg/ℓ

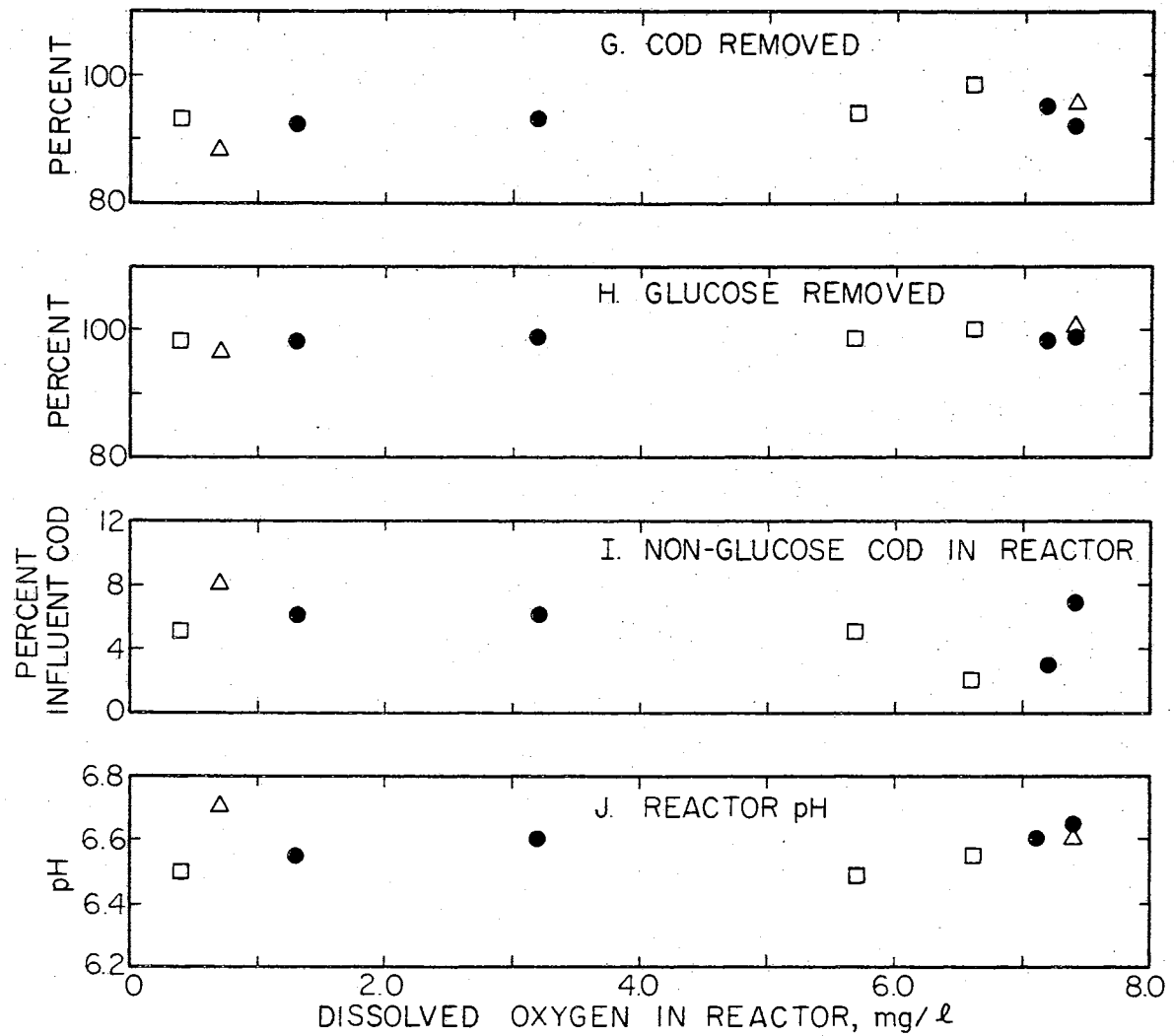


Figure 42 - Effect of dissolved oxygen concentration on various parameters during steady state operation. (Symbols used are the same as in Figure 41.)

since even at the lowest steady DO level studied, only 5 per cent of the influent COD was transformed into non-glucose compounds. Also, metabolic intermediates and endproducts accounted for a somewhat higher (7 per cent) portion of the feed COD at the highest steady state DO level (7.4 mg/l) studied (Experiment III). Some comments are warranted on the nature and extent of release of metabolic intermediates and end-products. The minimum and maximum amounts of intermediates detected during the steady state operations have been shown earlier in Table V.

Acetic acid occurred randomly during the steady state operations at low and high DO levels. It is interesting to note that the occasional release of this volatile acid did not depend upon the oxygen tension of the medium in the range of 0.4 to 7.4 mg/l. Somewhat higher concentrations of acetic acid were observed in two samples during steady state operation at a DO level of 0.4 mg/l and a feed concentration of 2130 mg/l glucose COD (Table V). However, when these results are compared with the maximum quantity of acetic acid released during steady state operation at the highest DO level (Experiment III) and lower organic loading, it can be concluded that DO did not influence this phenomenon. Furthermore, if the lower levels of dissolved oxygen concentration affected the metabolic activities of the organisms resulting in partial oxidation of glucose, then all of the samples taken during the steady state operation at these DO levels would have contained acetic and possibly other volatile acids. On the contrary, the total amount of metabolic intermediates, and the near absence of acetic acid in most of the samples during steady state operations at DO levels of 0.4 and 0.7 mg/l, argue against the possibility of oxygen limitation. The production of volatile acids (particularly acetic acid)

during aerobic metabolism of carbohydrates has been observed by various workers for both heterogeneous and pure culture systems (18)(57)(61)(67)(73)(116)(118)(119).

Smaller amounts of keto acids and ribose were detected in the medium during operation at high and low DO tensions. The slightly higher concentration of ribose and organic nitrogen in the effluents at DO levels of 0.4 and 0.7 mg/l might be due to the continued lysis of a portion of the population initiated earlier during the secondary response after the shock loading. The presence of ribose (at all times) and organic nitrogen (occasionally) at all DO levels suggest that partial cell lysis is a common phenomenon for all systems, irrespective of the existing DO levels.

The pH of the medium was relatively constant (6.5 to 6.7) during steady state operations at the different DO levels studied. The pH of the feed was 7.0 and the decrease observed can be attributed to the removal of ammonia nitrogen for growth (to be discussed later in this report). Since the ratio of COD consumed to ammonia nitrogen used did not vary significantly at the DO levels studied (Figure 41D); the steady state pH values remained relatively constant.

In Figure 41A it is seen that the respiration rate in the steady state before shock loadings were applied was essentially constant and independent of dissolved oxygen concentration; these findings are in agreement with those of Rickard and Gaudy (95). Respiration rates were somewhat lower in steady states developed after application of the shock, and after the shock had been removed, probably as a result of the change in predominance which occurred in response to the increase in feeding level. Dissolved oxygen concentration had no effect on

respiration rate, over the range studied. Critical DO levels, below which respiration is affected, have been reported for various organisms in the range of 0 to 0.7 mg/l (40)(87)(88)(89). The present study indicates that the value would in all probability lie somewhat below 0.4 mg/l. Also, it seems doubtful that any of the values thus far reported for various pure culture studies would be directly translatable to activated sludge or heterogeneous populations, since lower DO levels would tend to select organisms which had lower critical concentrations.

The percentage of substrate channelled into respiration is shown in Figure 41B. Values for this parameter ranged between 42 and 53 per cent, and dissolved oxygen concentration had no effect. Similar findings have been reported by van Gils (94).

Cell yields at the various DO levels are shown in Figure 41C. Before shock loads were applied, the yields varied from 32 to 38 per cent over the range of dissolved oxygen concentration studied. The results are in general agreement with those of Rickard and Gaudy (95). The steady state yield values after the shocks were applied were higher, due probably to the changes in species predominance which took place. In general, cell yield was not affected by dissolved oxygen concentration. The results are in accord with those of Phillips and Johnson (88), who found no correlation between oxygen supply rates and cell yield if the O_2 tension was maintained above 0.01 to 0.02 atmospheres (approximately 0.4 to 0.7 mg/l at 30^{o}C).

Figure 41D shows that the amount of COD removed per weight of ammonia nitrogen consumed varied little with DO level, remaining at approximately 20 in the DO range studied, and this value is in agreement with that reported by Sawyer (21). It is noted that, in the

present study, the COD:N ratio in the feed was maintained at approximately 10:1 in order to ensure that nitrogen source would not be a limiting factor, and there were always appreciable quantities of unused nitrogen source in the effluent.

The protein and carbohydrate contents of the cells during steady state operation are shown in Figures 41E and 41F, respectively. The protein content varied from 36 to 51 per cent, and this variation was independent of dissolved oxygen concentration over the range 0.4 to 7.4 mg/l DO. Rickard and Gaudy (95) had previously shown that over a DO range of 1.4 to 7.1 mg/l, protein content was unaffected by dissolved oxygen concentration. In this study they did observe a rather sharp drop in carbohydrate content as the DO level increased. In the present study (Figure 41F) there was only a slight trend toward lower carbohydrate values at the higher DO levels.

In general, the values for the parameters studied under steady state conditions for dissolved oxygen concentrations ranging from 0.4 to 7.4 mg/l were independent of dissolved oxygen concentration.

2. Effect of Dissolved Oxygen on Transient Response

It is interesting to compare the transient responses at the various DO levels with respect to substrate removal after an increase in feed concentration. Such a comparison is facilitated in Figure 43. Zero on the time axis represents the time of changing the feed. Also plotted are the theoretical dilute-in curves for the shock loads. These curves represent the concentration of COD which would have been found in the reactor if the cells had continued to use the previous steady state concentration of feed but had not been able to remove the added feed in the shock loading. Experiments conducted at high initial

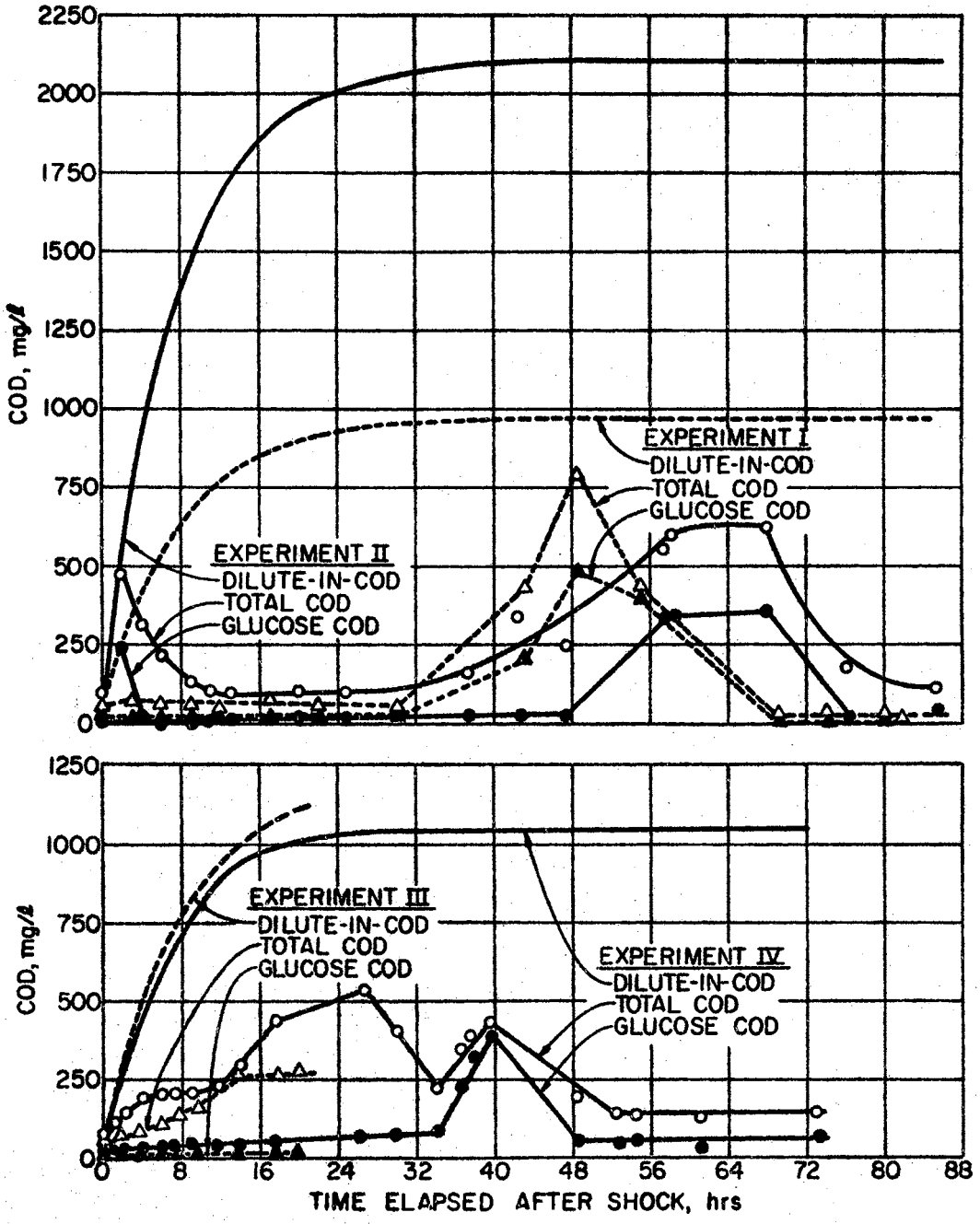


Figure 43 - Transient response to shock loads at various DO levels.

DO are shown in the upper portion of the figure. At the high DO level, immediate substrate leakage occurred at the triple loading. The secondary response to the higher shock loading occurred later than the secondary response to the lower shock loading. The responses for Experiments I, III, and IV can be compared directly. All were shocked to approximately the same extent (1000 mg/l feed changed to 2000 mg/l). The only difference in the systems was the DO level and naturally-occurring differences in species predominance which may have existed in the microbial populations. At the highest DO level, the initial response (the metabolic response) was an entirely successful one, i.e., there was no glucose or COD leakage in the effluent. At the lower DO level, the initial response was less successful. At the intermediate DO level (Experiment III), the run was terminated 20 hours after the shock, before a secondary response might have occurred. However, at the lowest DO level, a lower concentration of COD appeared in the effluent during the secondary response (change in predominance) than at the higher DO level. The results indicate that at lower DO tensions there may be need for concern regarding the initial response. In this regard the results are somewhat difficult to interpret because in all systems using heterogeneous populations the only control over the type of species is that provided by the enrichment culture principle, and it cannot be said that the predominating organisms in Experiments III and IV would not have given rise to COD leakage at a higher DO level. The fact that COD leakage during the early phases of the response occurred even at the DO level of 3.2 (Experiment III) tends to indicate that DO level was not the controlling factor. It is noted that in systems III and IV, leakage of COD during the early part of the response

was due not to nonmetabolism of the feed substrate, but to the accumulation of metabolic products, largely acetic and pyruvic acids. During the early phase of the response, no change in predominance was evident by microscopic examination. This early phase of the transient response is therefore attributed to the metabolic or physiological response of the indigenous population to the change in feed concentration.

The accumulations of acetic and pyruvic acids during the early response to shock loads at high and low dissolved oxygen concentrations (Experiments II and IV) might be due to the immediate biochemical response of the existing organisms to the increased availability of glucose. The immediate metabolic response within the cells could involve an increased rate of glycolysis and also the repression of the enzymes required for the utilization of acetate and pyruvate. These biochemical mechanisms have been discussed earlier.

Although the accumulations of acetic and pyruvic acids during the early response might appear to be correlated with DO level in Experiments I, II, and IV, consideration of all data obtained during transient and steady state operations in all four experiments shows that accumulations of these acids may occur even at high DO levels.

Considerable amounts of pyruvic acid (200 mg/l) were released during the secondary response of the populations in Experiment II. The DO level in the reactor during this period was 7 mg/l. Somewhat higher amounts of pyruvic acid (380 mg/l) were released by the cells during the secondary response in Experiment IV. The DO during this period was in the range of 0.7 to 1.2. It is significant to note that maximum production of pyruvic acid in either system occurred during the partial washout of the population (secondary response). Therefore it

seems likely that the large accumulations of pyruvic acid by the cells during the secondary response were associated more with the complex interactions between the organisms after the shock load than with the DO level in the range studied. This is further evident from the absence of pyruvic acid during steady state operations at the different DO concentrations.

The devlopment of a secondary transient response in these systems was particularly interesting because it represented in each case a severe loss of biological solids and substrate, and it was associated with an ecological shift or change in microbial predominance. This secondary response occurred in Experiments I, II, and IV (Experiment III was terminated early), and therefore is not an effect of dissolved oxygen concentration. The period of depressed solids and high substrate leakage usually corresponded to the highest organic nitrogen content in the effluent. A sudden rise of organic nitrogen from zero to 27 mg/l in Experiment I, 4 to 28 mg/l in Experiment II, and from zero to 24 mg/l in Experiment III was observed during the secondary response. Increased amounts of ribose were present in the effluents of these experiments during this period. This phenomenon indicated that the secondary response was associated with some degree of lysis of the population.

It seems reasonable to expect that the initial response to the higher substrate concentration would, within the capability of the cells, involve a biochemical or physiological mechanism of the indigenous cells and that an ecological shift (if it occurred) would follow the more immediate feeding pressure placed upon the system. The physiological response involves a transient increase in growth rate.

The logarithmic growth rate constant which is, in the steady state prior to the shock, held equal to the dilution rate, is less restricted as a greater amount of substrate is admitted into the system, and the growth rate constant rises in accord with the increase in food supply (the essence of a successful response). However, in the presence of this more abundant supply of substrate, the results indicate that two phenomena, which are also observed in batch experiments, occur. The cells have a tendency to store carbon source, as evidenced by a rise in carbohydrate content (this phenomenon occurred in Experiments I, III, and IV), and there is a tendency for elaboration of metabolic products. These facts provide indication that the cells can dissimilate the original exogenous carbon source at a greater rate than that at which they can channel the carbon source into synthesis of daughter cells. This response was not related to the dissolved oxygen concentration, since it occurred at both high and low DO tensions.

The above analysis does not provide an explanation for the decrease in solids and concomitant rise in effluent COD after the primary response. This secondary response was of such severity that it cannot be considered to be simply an "overshoot" of the primary response. Explanation for this rather severe dilute-out of cells (secondary response) may involve complex antagonistic relationships among members of the population (e.g., production of substances which may be inhibitory to other cells). Also, it is known from the work of George and Gaudy (120) that fairly small decreases in the pH can cause some cells to dilute out of the system. In the present experiments, slight (but noticeable) decreases in pH occurred (except for Experiment I) as a result of accumulation of acid metabolic products. These

slight changes in pH may help account for cell dilute-out which precipitated the secondary response. It is known that volatile fatty acids show growth-inhibiting and killing actions on certain species of bacteria (145)(146). The toxicity of the fatty acids has been found to increase with the concentration of the non-ionized acid (lower pH) (146). Wolin (146) observed that addition of 45 mM acetic acid to a growth medium caused stimulation of the growth of Escherichia coli at pH 7.0, but when the initial pH of the medium was 6.5, severe growth inhibition (41 per cent) ensued. Eighty per cent growth inhibition was noted at a pH of 6.0.

Changes in the predominating populations of a steady state system due to the gradual shock loading of sodium chloride in the feed (glucose) have been reported by Kincannon (138). These changes were found to result in cyclic variations in effluent COD and biological solids before the attainment of a new steady state.

B. Short-term Batch Experiments

1. Metabolism of Glucose

The results obtained with the growing and nonproliferating systems show that the kinetics of COD removal and solids growth depend upon the initial COD:biological solids ratio employed. Under growing conditions a decrease in the ratio of COD:solids (increase in initial solids) shifts the kinetics of COD removal and solids growth from increasing first-order (logarithmic or exponential rate) to zero-order. In the absence of exogenous nitrogen, a decrease in COD:solids ratio can change the kinetics of COD removal from decreasing first-order to zero-order reaction kinetics. The different phases of first order reaction noted in the nonproliferating systems might be due to the different

rates at which the metabolic intermediates are utilized or to the sequential development of different populations.

The summaries of data for the experiments on glucose are presented in Tables XXVI and XXVII. Table XXVI shows the initial conditions and the duration of the substrate removal phase in each system. The initial cell composition is also shown. Table XXVII summarizes the characteristics of each system at the time of removal of the substrate. The net amounts of cell components synthesized are reported as percent of the total sludge synthesized and also as percent of the COD removed (yield).

Sludge (cell) yield in the growing systems did not depend upon the initial biological solids concentrations (Table XXVII). At lower initial solids concentrations (Experiments I and II) the nonproliferating system gave lower yields than did the corresponding growing systems. There was, however, essentially no difference in solids accumulation between the two systems when a higher initial cell concentration was employed (Experiment III). This is due to the increased conversion of the substrate COD into sludge carbohydrate in the nonproliferating system. As seen in Table XXVII, as much as 58 per cent of initial COD was diverted into carbohydrate synthesis in the nonproliferating system when a higher initial concentration of sludge was employed (Experiment III). It is also seen that the nonproliferating cells in Experiment I synthesized as much as two and one-half times their weight of carbohydrate. It appears, therefore, that while the capacity of the cells to synthesize carbohydrates is limited, this capacity can be quite high. Similar effect of initial solids concentration on the conversion of substrate COD to cell carbohydrate was noted in the growing systems.

TABLE XXVI

SUMMARY OF INITIAL CONDITIONS AND DURATION OF SUBSTRATE REMOVAL IN GLUCOSE SYSTEMS

Exp. No.	Fig. No.	Initial Conditions of System: COD Biological Solids	COD mg/l	Biological Solids mg/l	Sludge Carbohydrate mg/l	Sludge Carbohydrate Percent Dry Wt.	Sludge Protein mg/l	Sludge Protein Percent Dry Wt.	Sludge Lipids mg/l	Sludge Lipids Percent Dry Wt.	Duration of Substrate Removal Phase Hrs:Min
					Growing System						
I	8	7.5	2520	336	150	44.6	70	20.8	66	19.6	13;15
II	10	2.8	2120	758	161	21.2	205	27.0	-	-	6;40
III	12	1.3	1720	1300	427	32.8	340	26.2	-	-	3:00
					Nonproliferating System						
I	8	7.5	2540	340	150	44.1	80	23.5	66	19.4	52:00
II	10	2.8	2110	746	161	21.6	215	28.8	-	-	18:00
III	12	1.3	1730	1310	455	34.7	344	26.3	-	-	3:00

TABLE XXVII

SUMMARY OF CHARACTERISTICS OF GLUCOSE SYSTEMS AT THE TIME OF REMOVAL OF EXOGENOUS SUBSTRATE

Exp. No.	Fig. No.	Time Hrs:Min	COD		Sludge			Sludge Carbohydrate					Sludge Protein					Sludge Lipid				
								Total		Synthesis			Total		Synthesis			Total		Synthesis		
			Residual mg/l	Removed mg/l	Total mg/l	Synthesis mg/l	Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Sludge Synthesized	Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Sludge Synthesized	Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Sludge Synthesized	Yield Percent
								Growing System														
I	8	13:15	76	2444	1880	1544	63.2	885	47.1	735	47.6	30.0	420	22.3	350	22.7	14.3	110	5.9	44	2.8	1.8
II	10	6:40	160	1960	1934	1176	60.0	930	48.1	769	65.4	39.2	490	25.3	285	24.2	14.5	-	-	-	-	-
III	12	3:00	87	1633	2340	1040	63.7	1140	48.7	713	68.6	43.7	530	22.6	190	18.3	11.6	-	-	-	-	-
								Nonproliferating System														
I	8	52:00	400	2140	1470	1130	52.8	1070	72.8	920	81.4	43.0	88	6.0	8	0.7	0.4	140	9.5	74	6.5	3.3
II	10	18:00	240	1870	1625	879	47.0	678	41.7	517	58.8	27.6	260	16.0	45	5.1	2.4	-	-	-	-	-
III	12	3:00	96	1634	2400	1090	66.7	1400	58.2	945	86.7	57.8	390	16.3	46	4.2	2.8	-	-	-	-	-

The yield of sludge protein in all growing systems was considerably lower than that of carbohydrate. It should be noted that the values for total protein reported in Table XXVII represent the concentration at the time of removal of exogenous substrate and not the maximum synthesized in the systems. In all growing systems, synthesis of the protein attained maximum values during the early endogenous phase involving simultaneous decrease of sludge carbohydrate. These results are in agreement with those reported by Gaudy and Engelbrecht (15), Komolrit, Goel, and Gaudy (7), and Krishnan and Gaudy (147). The latter employed initial COD:solids rations which were approximately in the range used in Experiments II and III of the present study.

Walters, Engelbrecht, and Speece (114) have reported that the storage of carbohydrate by a heterogeneous population grown on glucose and yeast extract amounted to 40 per cent of the initial substrate COD in a range of "food:microorganisms" (COD:solids) ratios between 0.73 and 4.3. At ratios higher than 4.3, they found a rapid decrease in the amount of substrate COD converted into carbohydrate. However, in the present study there was a progressive decrease in carbohydrate yield with increasing COD:solids ratio. This may be due to the fact that the removal of substrate at lower initial solids concentrations depends more upon balanced growth by replication rather than upon carbohydrate storage (147)(148).

Metabolism of glucose by growing as well as nonproliferating cells did not involve accumulation of lipid. It can be seen from Table XXVII that the synthesis of carbohydrate and protein accounted for 70 per cent of the net solids increase in the growing system of Experiment I, and lipid synthesis contributed only three per cent. Similar

findings have been reported by van Gils (94).

The percent of substrate respired by the cells was very low in the growing and nonproliferating systems. About 15 and 13 per cent respiration were observed in Experiments II and III, respectively. Similar results were reported by other workers (15)(93)(94)(109). The extent of respiration was not altered in the absence of nitrogen in Experiment III, but somewhat higher respiration was noted in Experiment II.

The amounts of metabolic intermediates and endproducts released by the growing and nonproliferating cells metabolizing glucose are presented in Table XXVIII. Examination of these data shows that there is no strict correlation between the initial COD:solids ratios and the amounts of metabolic intermediates in the growing system. However, this is not true in the nonproliferating system where a tendency for decreased production of intermediates was noted at lower COD:solids ratios.

It is interesting to note that glucose can be metabolized completely even by a nonproliferating system with a high initial COD: solids ratio (Experiment I, Figure 8), provided the period of contact is long. Since glucose is known to be dissimilated by certain organisms at a much more rapid rate than is commensurate with the growth rate and total crop of cells (58)(59), and also since the catabolism of substrates need not necessarily be coupled to the energy-consuming biosynthetic reactions (22), it is not surprising to find a rapid catabolism of glucose even under nonproliferating conditions. Under growing conditions, the intermediary metabolites produced during the catabolism of glucose can be effectively utilized for the synthesis of various cell components, and hence there should be less necessity for

TABLE XXVIII

MAXIMUM AMOUNTS OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS RELEASED DURING METABOLISM OF GLUCOSE

Exp. No.	Fig. No.	Initial COD / Initial Biological Solids	Maximum Amounts of Intermediates and/or Endproducts	
			COD mg/l	As Percent Initial COD
		Growing System		
I	8	7.5	326	12.9
II	10	2.8	170	8.0
III	12	1.3	139	8.1
		Nonproliferating System		
I	8	7.4	785	30.8
II	10	2.8	470	22.3
III	12	1.3	250	14.5

secretion of the metabolic intermediates into the external medium. Under nonproliferating conditions, however, the intermediates cannot be utilized for the synthesis of macromolecular components such as RNA, DNA, and protein. In the absence of synthesis of such vital compounds, these intermediates can serve as substrates for the synthesis of storage products like carbohydrates. When a small initial inoculum of cells is employed, carbohydrate synthesis is limited, not by the availability of substrate, but by the amount of enzyme initially present in the system. The larger extracellular accumulation of metabolic intermediates in the system with lower initial solids is more indicative of a relatively lower rate of carbohydrate synthesis than of the rate of utilization of exogenous glucose.

Another possible reason for the release of metabolic intermediates may be the repression of TCA cycle enzymes by glucose (55)(56)(62)(63)(64)(65) as evidenced by the presence of acetic and pyruvic acids in the medium. These acids are released under both growing and nonproliferating conditions, thus indicating that repression may occur regardless of the presence of exogenous nitrogen. Under nonproliferating conditions, the quantities of these acids were somewhat higher. This may be due to the reduced role of TCA-like cycles during the metabolism of glucose in the absence of nitrogen. The anabolic reactions that function in the supply of five-carbon skeleton (α-ketoglutarate) which is required for the biosynthesis of glutamate and amino acids derived from glutamate are not active in the absence of exogenous nitrogen. Therefore, the major function of, for example, the TCA cycle under this circumstance is the production of energy by the catabolic sequence of reactions. Further, the energy requirement

for carbohydrate synthesis is lower than that for the synthesis of protein and nucleic acids. On the other hand, the flow of glucose through the glycolytic pathway is not restricted by the pace of TCA cycle reactions. It has been reported that even during balanced growth, the glycolytic reactions proceed much more rapidly than do the terminal oxidative reactions of the TCA cycle (61)(63). The difference in the relative rates of the glycolytic pathway and the TCA cycle reactions can therefore be much accentuated during the metabolism of glucose in the absence of nitrogen.

2. Metabolism of Sucrose

The summary of data on the cell composition and synthesis of protein and carbohydrate during the metabolism of sucrose is presented in Tables XXIX and XXX.

The sludge yields obtained in these experiments were comparable to those reported for glucose (Table XXVII). The yield of cell carbohydrate was somewhat lower than obtained in the glucose system with a comparable initial COD:solids ratio (Experiment II, Table XXVII). As in the glucose systems, carbohydrate synthesis was much higher under nonproliferating conditions. In Experiment III, the nonproliferating cells of the sucrose system (Figure 15) did not synthesize significantly higher amounts of carbohydrate than did the growing system; the cells in Experiment II (Figure 14) which had the same initial COD: solids level did do so. The reason for this anomalous behavior is not understood. Similar behavior was also noted in the nonproliferating system of Experiment II on Glucose (Table XXVII, and Figure 10). Any explanation suggesting "saturation" of cell capacity for storage of carbohydrate can be ruled out in these cases, since it was shown that

TABLE XXIX

SUMMARY OF INITIAL CONDITIONS AND DURATION OF SUBSTRATE REMOVAL IN SUCROSE SYSTEMS

Exp. No.	Fig. No.	Initial Conditions of System: COD Biological Solids	COD mg/l	Biological Solids mg/l	Sludge Carbohydrate mg/l	Sludge Carbohydrate Percent Dry Wt.	Sludge Protein mg/l	Sludge Protein Percent Dry Wt.	Duration of Substrate Removal Phase Hrs:Min
				Growing System					
II	14	2.4	2000	835	338	40.5	270	32.3	4:25
III	15	2.2	1760	787	270	34.3	400	50.8	5:20
				Nonproliferating System					
II	14	2.5	2150	855	350	40.1	250	29.2	4:25
III	15	2.4	1815	750	225	30.0	388	51.7	10:00

TABLE XXX

SUMMARY OF CHARACTERISTICS OF SUCROSE SYSTEMS AT THE TIME OF REMOVAL OF EXOGENOUS SUBSTRATE

Exp. No.	Fig. No.	Time Hrs:Min	COD		Sludge			Sludge Carbohydrate					Sludge Protein				
								Total		Synthesis			Total		Synthesis		
			Residual mg/l	Removed mg/l	Total mg/l	Synthesis mg/l	Synthesis Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Sludge Synthesized	Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Sludge Synthesized	Yield Percent
								Growing System									
II	14	6.00	103	1897	1980	1145	60.4	825	41.7	487	42.5	25.6	550	27.8	280	24.5	14.8
III	15	5.20	130	1630	1910	1123	68.9	785	41.1	515	45.9	31.6	570	29.8	170	15.1	10.4
								Nonproliferating System									
II	14	6.00	280	1870	1900	1045	55.9	1220	64.2	870	83.3	46.5	320	16.8	70	6.7	3.7
III	15	10.00	405	1410	1550	800	56.7	725	46.8	500	62.5	35.5	485	31.3	97	12.1	6.9

nonproliferating cells (glucose system Experiment I, Figure 8) were capable of synthesizing carbohydrate to the extent of more than two times their own dry weight.

A noteworthy characteristic of the sucrose systems was the very rapid degradation of the substrate in the presence or absence of exogenous nitrogen (Figures 13, 14, and 15). This rapid metabolism resulted also in the quantitative conversion of most of the sucrose into its constituent monosaccharides. The appearance of large amounts of glucose and fructose in the external medium during the rapid metabolism of sucrose suggests that either sucrose was hydrolyzed extracellularly, or the intracellular hydrolysis of the disaccharide occurred at a much faster rate than the utilization of the hydrolytic products by the cell, and they were subsequently excreted. The initial breakdown of sucrose may involve either its hydrolysis into glucose and fructose by the action of the enzyme, invertase, or its phosphorylysis to form glucose-1-P and fructose (149). Invertase can act on sucrose either intracellularly or extracellularly (150)(151). It has been speculated that certain potent enzymes like invertase can be liberated from the cells during lysis of a portion of the population, a phenomenon considered to be a sacrifice by the lysing cells in order to allow the survival of the remainder (152).

It is seen from Figures 14 and 15 that the initial accumulation of glucose is slightly higher than that of fructose. This might be due to the conversion of a part of the fructose liberated inside the cell into a form not readily diffusible from the cell, or to a direct conversion of some fructose into glucose. Such a direct conversion of fructose into glucose has been observed in some animal tissues (153) and is

thought to occur in bacteria (154).

Acetic acid was released by cells in both systems during the initial period of metabolism of glucose. Since both glucose and fructose may be metabolized via the glycolytic pathway, the production of acetic acid might be due to the reasons discussed earlier under glucose metabolism.

Percent theoretical oxygen demand exerted at the time of maximum solids growth and COD removal amounted to 22.3 per cent in the growing system and 15.1 per cent in the nonproliferating system. Wuhrmann (93) has reported 13.5 per cent oxidation of sucrose by resting cells of activated sludge not acclimated to sucrose.

3. Metabolism of Lactose

Metabolism of lactose involves the action of β-galactosidase and β-galactoside permease, the enzymes which are induced during growth on lactose (155). The results obtained with lactose in the present study indicate that lactose is hydrolyzed intracellularly, and the monosaccharide moieties, glucose and galactose, are not excreted by the cells under growing or nonproliferating conditions. Small amounts of galactose were detected in the medium of both systems (Figure 17, Table XII) at the beginning of the experiment. This was probably due to the presence of small amounts of galactose as an impurity in the lactose used. If the disaccharide were broken down extracellularly or if glucose interfered with the utilization of galactose inside the cell, it could be expected that much larger amounts of galactose would have been detected in the medium. Adhya and Echols (156) have reported that glucose did not "substantially" inhibit induction of the galactose enzymes when glucose and galactose were produced intracellularly by

β-galactosidase hydrolysis of lactose in Escherichia coli.

It was seen in Table XII that very small amounts of intermediates were detected in the growing system. However, under nonproliferating conditions, large amounts of acetic and keto acids were found in the medium. Since the degradation of galactose involves its initial conversion to UDP-glucose and channelling into the glycolytic pathway, the same kinds of intermediates as produced during the metabolism of glucose can be expected to accumulate. The presence of keto acids in the nonproliferating system could be due to the lack of utilization of the keto acid intermediates of the TCA cycle for protein synthesis (as discussed earlier).

4. Metabolism of Fatty Acids

The initial conditions of the fatty acid systems are given in Table XXXI. The data on the synthesis of biological solids, carbohydrate, and protein are summarized in Table XXXII.

a. Metabolism of Acetic Acid

The results of the experiments conducted under growing and nonproliferating conditions with sludge acclimated to a low initial pH (4.5) were shown in Figure 18. It was seen that the cells were able to metabolize the added acetic acid even at low pH. Since the cells had been acclimated, prior to the experiment, to a feed of low pH, organisms able to thrive under acid conditions would be expected to have gained predominance in the stock reactor.

It was observed earlier that a drop in pH of the continuous flow system might have been the cause for the dilute-out of a portion of the cells. Some workers (145)(146) have reported a toxic effect of volatile fatty acids on certain organisms in a medium at lower pH. However,

TABLE XXXI

SUMMARY OF INITIAL CONDITIONS AND DURATION OF SUBSTRATE REMOVAL IN FATTY ACID SYSTEMS

Substrate	Exp. No.	Fig. No.	Initial Conditions of System: COD Biological Solids	COD mg/l	Biological Solids mg/l	Sludge Carbohydrate mg/l	Sludge Carbohydrate Percent Dry Wt.	Sludge Protein mg/l	Sludge Protein Percent Dry Wt.	Duration of Substrate Removal Phase Hrs:Min
					Growing System					
Acetic Acid	I	18	10.2	2060	201	90	44.7	81	40.3	37:20
Acetic Acid	II	20	9.9	950	96	13	13.5	35	36.5	08:15
Acetic Acid	III	22	3.6	2040	570	131	23.0	174	30.5	06:10
Sodium Acetate	I	23	4.7	2000	425	26	6.1	197	46.4	17:15
Sodium Acetate	II	24	3.4	940	275	69	25.1	113	41.1	05:45
Propionic Acid	I	25	15.2	5050	332	65	19.6	100	30.1	75:35
Propionic Acid	II	27	5.0	3180	632	190	30.1	190	30.1	11:40
					Nonproliferating System					
Acetic Acid	I	18	11.4	2050	180	73	40.6	63	35.0	37:20
Acetic Acid	II	20	8.6	925	108	16	14.8	35	32.4	15:00
Acetic Acid	III	22	3.3	2040	610	140	23.0	200	32.8	10:10
Sodium Acetate	I	23	4.7	2050	440	25	5.7	219	49.8	17:15
Sodium Acetate	II	24	3.1	1000	320	80	25.0	131	40.9	09:15
Propionic Acid	I	25	15.6	5150	330	65	19.7	100	30.3	105:45
Propionic Acid	II	27	5.8	3430	590	225	38.1	190	32.2	26:55

TABLE XXXII

SUMMARY OF CHARACTERISTICS OF FATTY ACID SYSTEMS AT THE TIME OF REMOVAL OF EXOGENOUS SUBSTRATE

Substrate	Exp. No.	Fig. No.	Time Hrs:Min	COD Residual mg/l	COD Removed mg/l	Sludge Total mg/l	Sludge Synthesis mg/l	Sludge Synthesis Yield Percent	Sludge Carbohydrate Total mg/l	Sludge Carbohydrate Total As Percent Sludge Dry Wt.	Sludge Carbohydrate Synthesis mg/l	Sludge Carbohydrate Synthesis As Percent Sludge Synthesized	Sludge Carbohydrate Synthesis Yield Percent	Sludge Protein Total mg/l	Sludge Protein Total As Percent Sludge Dry Wt.	Sludge Protein Synthesis mg/l	Sludge Protein Synthesis As Percent Sludge Synthesized	Sludge Protein Synthesis Yield Percent
											Growing System							
Acetic Acid	I	18	37:20	298	1762	962	761	43.2	255	26.5	165	21.7	9.4	215	22.3	134	17.6	7.6
Acetic Acid	II	20	08:15	49	901	535	439	48.7	105	19.6	92	21.0	10.2	150	28.0	115	26.2	12.8
Acetic Acid	III	22	06:10	72	1968	1250	680	34.6	230	18.4	99	14.6	5.0	330	26.4	156	22.9	7.9
Sodium Acetate	I	23	17:15	300	1700	1155	730	43.0	56	4.8	30	4.1	1.8	502	43.5	305	41.8	17.9
Sodium Acetate	II	24	05:45	27	913	755	480	52.6	146	19.3	77	16.0	8.4	361	47.8	248	51.7	27.2
Propionic Acid	I	25	75:35	240	4810	2080	1748	36.3	212	10.2	147	8.4	3.1	540	26.0	440	25.2	9.1
Propionic Acid	II	27	11:40	160	3020	1835	1203	39.8	475	25.9	285	23.7	9.4	630	34.3	440	36.6	14.6
											Nonproliferating System							
Acetic Acid	I	18	37:20	152	1898	985	805	42.4	600	60.9	527	65.5	27.8	90	9.1	27	3.4	1.4
Acetic Acid	II	20	15:00	520	405	340	232	57.3	165	48.5	149	64.2	36.8	-	-	-	-	-
Acetic Acid	III	22	10:10	232	1809	1230	620	34.3	400	32.5	260	41.9	14.4	180	14.6	nil	-	-
Sodium Acetate	I	23	17:15	1740	310	←		→	System failed to remove exogenous substrate					→				
Sodium Acetate	II	24	09:15	122	878	515	195	22.2	120	23.3	40	20.5	4.6	131	25.4	nil	-	-
Propionic Acid	I	25	105:45	5200	nil	←		→	System failed to remove exogenous substrate					→				
Propionic Acid	II	27	26:55	250	3180	1690	1100	34.6	750	44.4	525	47.7	16.5	217	12.84	27	2.5	0.8

the results obtained in this study show that lower pH per se need not be harmful to activated sludge treatment of acetic acid wastes in a batch reactor, provided the organisms are "trained" (or selected) to use the substrate at low pH. Brower and Gaddis (157) have reported effective removal of substrate (glucose + yeast extract) at low pH (2.4 to 3.0) in a medium with insufficient buffer capacity.

The peculiar feature of the growing system at low initial pH (Figure18) was the lower utilization of ammonia nitrogen during the substrate removal phase. As much as 63 mg/l of COD was removed for each mg/l of ammonia nitrogen consumed. This indicates a difference, probably, in the types of organisms present in the system at lower pH as compared to those in the system at higher pH (Experiment III, Figure 22), where a COD:nitrogen utilization ratio of only 36 was obtained.

The behavior of the nonproliferating system with low initial pH (Figure 18) can be compared to a similar system with increased buffer capacity (pH 6.7, acetic acid, Experiment II, Figure 20). Even though the latter system had almost the same initial COD:solids ratio, COD removal efficiency was drastically reduced. It should be noted that this system was run for only 15 hours. This lower rate of COD removal could not be attributed to the production of large amounts of extracellular metabolic intermediates observed in the system, since they were re-utilized by the cells. The accumulated intermediates, however, inhibited further removal of acetic acid until they were reduced to low levels.

Comparison of Experiments II and III (Figures 20 and 22) which were conducted using the same sludge acclimated to a pH of 6.7 shows

the effect of initial COD:solids ratio on the ability of the cells to utilize acetic acid under nonproliferating conditions. Even though the conversion of substrate into carbohydrate was less efficient in the system at a lower initial COD:solids ratio (acetic acid, Experiment III, Table XXXII), high COD removal was obtained in this system (compare Figures 20 and 22). Carbohydrate accounted for only 42 per cent of the net solids accumulated in this system. This suggests that synthesis of other storage products, e.g., lipid, might have contributed to the enhanced substrate utilization.

A significant feature of all acetic acid systems was the release of large amounts of metabolic intermediates and/or endproducts. Table XXXIII shows the maximum conversion of initial COD into non-acetate COD in the growing and nonproliferating systems.

It is seen from Table XXXIII that the conversion of original substrate into metabolic intermediates was much greater in the acetic acid systems than in the glucose systems (compare Tables XVIII and XXXIII) under either growing or nonproliferating conditions. Initial pH had no significant influence on the production of intermediary products in the growing systems (compare Experiments I and II). Higher amounts of intermediates were released in the nonproliferating system with an initial pH of 6.7 (Experiment II). In Experiments II and III, which were conducted with sludge from the same stock reactor and the same initial pH, lower amounts of intermediates were produced at the lower initial COD:solids ratio under growing as well as nonproliferating conditions.

Analyses made during Experiment I (lower initial pH) indicated the release of keto acids during metabolism of acetic acid in the presence

TABLE XXXIII

MAXIMUM AMOUNTS OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS RELEASED DURING METABOLISM OF ACETIC ACID

Exp. No.	Fig. No.	Initial pH	Initial COD / Initial Biological Solids	Maximum Amounts of Intermediates and/or Endproducts	
				COD mg/l	As Percent Initial COD
			Growing System		
I	18	4.5	10.2	635	30.8
II	20	6.7	9.9	342	36.0
III	22	6.7	3.6	278	13.6
			Nonproliferating System		
I	18	4.5	11.4	795	38.8
II	20	6.7	8.6	457	50.6
III	22	6.7	3.3	621	30.4

or absence of exogenous nitrogen. It is known that for growth of microorganisms on acetate as sole carbon source, two adaptive enzymes which constitute the glyoxylate bypass, malate synthetase and isocitrate lyase, must be induced (158). It is possible that glyoxylate, which is a key intermediate during the metabolism of acetic acid and which may lead to a pathway for the synthesis of cellular carbohydrate from acetate (159) accumulated at a higher rate than it could be utilized for the synthesis of carbohydrate components of the cell, and consequently was secreted by the cells.

b. Metabolism of Sodium Acetate

Figure 23 showed the response of the sodium acetate systems with the initial pH of 7.2. It was seen that the pH increased rapidly during the metabolism of sodium acetate under both conditions. Although the growing system eventually removed the substrate, the nonproliferating system failed to metabolize the substrate. The increase in pH was due to the removal of acetate and liberation of the sodium ions. Phosphate buffers are not effective in the range between 7.2 and 8.5 (160). The oxidation of sodium acetate can be written as follows (160):

$$2Na\ (C_2H_3O_2) + 4\ O_2 \longrightarrow Na_2CO_3 + 3CO_2 + 3H_2O$$

The higher pH would also have accelerated the stripping of ammonia from the medium of the growing system. This experiment points out the adverse effect of using salts of fatty acids as substrates without adequate buffering capacity in the medium. The growing systems of Experiments I and II (Figures 23 and 24) can be compared to see the effect of pH on the substrate removal performance of the systems. There was only a small difference in the initial COD:solids ratio between the two systems, but the substrate removal efficiency of the system with increased

buffering capacity (Experiment II, Figure 24) was far higher than that of the system with lower buffering capacity (Experiment I, Figure 23). The nonproliferating system of Experiment II (Figure 24) operated successfully.

It is interesting to compare the efficiency of sodium acetate utilization (Experiment I, Figure 23) with the utilization of acetic acid in Experiment I (Figure 18), in which the initial pH was very low. Even though the initial COD:solids ratio was very high in the latter system, it operated successfully even in the absence of exogenous nitrogen. From these studies it is evident that alkaline pH is more harmful to acetate removal by activated sludge than acid pH.

A significant aspect of the metabolism of sodium acetate and acetic acid is the high oxidation of greater proportions of these substrates and the correspondingly lower sludge yields as compared to the carbohydrate (glucose and sucrose) systems discussed earlier. Similar results have been reported by Placak and Ruchhoft (110), by van Gils (94), and by Burkhead and McKinney (161).

c. Metabolism of Propionic Acid

The results shown in Figure 25 for Experiment I using propionic acid are somewhat different from those obtained for the acetic acid system at the same low initial pH, 4.5. An unusually long lag period was noted prior to exponential growth on propionic acid. It should be noted that the cells employed in this system had been acclimated to a feed of 2000 mg/l propionic acid at pH 5.2. The feed level during the experiment was, however, above 3000 mg/l at pH 4.5, and therefore might have constituted a significant "shock load" to the cells. Also, the initial COD:solids ratio was very high, 15.6. The lag phase might

therefore represent a period in which enough enzymes were accumulated within the cells before initiation of exponential growth.

In Experiment I, substrate was not removed in the nonproliferating system even after a prolonged period of aeration. The behavior of this system differs from the nonproliferating system with acetic acid at low initial pH (Experiment I, Figure 18) wherein efficient substrate removal was observed. One reason for the failure of the propionic acid system could be the inability of this system to convert the substrate into non-nitrogenous storage materials. Also, the system apparently failed to oxidize the substrate. It can be seen from Table XXIII that the efficiency of conversion of substrate COD into carbohydrate in the system growing on propionic acid (Experiment I) was only one-third of that found in the system growing on acetic acid at the same low initial pH (Experiment I). Glyoxylate bypass enzymes might also be needed for the synthesis of carbohydrate storage material, and in the absence of an exogenous supply of nitrogen the nonproliferating cells could not induce these enzymes.

The response of the growing and nonproliferating systems in an adequately buffered medium was shown in Figure 27. The amount of buffer used was only slightly higher than used in the normal growth medium (Table I) on a COD:buffer basis. Growth occurred without a lag in both systems since the initial pH of the systems (5.3) was the same as that to which the cells had been acclimated; the enhanced rate of metabolism of propionic acid in these systems could be attributed to the higher initial biological solids concentration employed in this experiment. The higher initial biological solids level might have enabled the cells to accumulate higher amounts of storage products like carbohydrate

(compare carbohydrate yields in Experiments I and II, Table XXXII). This was evident from the results obtained with the nonproliferating system of Experiment II, where about 48 per cent of the solids accumulation was due to carbohydrate (Table XXXII). Since the growing system was able to synthesize appreciable amounts of carbohydrate, it can be expected that the cells employed in the nonproliferating system possessed the necessary enzymes for the synthesis of this material.

It was also noted that the cells in the nonproliferating system produced intermediary metabolites which reacted with anthrone reagent, suggesting an accumulation of carbohydrates. It seems probable that the cells released into the external medium some soluble carbohydrates. Secretion of loose slime, or extracellular gum, composed of polysaccharides has been reported with many organisms (162). Qualitative assessment of these carbohydrates present in the medium was not made.

C. Long-term Batch Studies

1. Metabolism of Glycerol

The summaries of data on the effect of extended aeration on the substrate removal and endogenous phases of growing and nonproliferating systems are given in Tables XXXIV, XXXV, and XXXVI.

Table XXXIV shows the initial conditions of the system and the duration of substrate removal and endogenous phases. The status of the endogenous phase at the time when the experiments were terminated is also stated in the last column.

Table XXXV shows the characteristics of the system at the time of removal of exogenous substrate. The weight of each component synthesized by the system per unit weight of COD removed is reported as percent yield of the component.

TABLE XXXIV

SUMMARY OF INITIAL CONDITIONS AND DURATION OF SUBSTRATE REMOVAL AND ENDOGENOUS PHASES OF GLYCEROL SYSTEMS

Exp. No.	Fig. No.	Initial Conditions of System: COD Biolog. Solids	COD mg/l	Biolog. Solids mg/l	Sludge Carbohydrate mg/l	Sludge Carbohydrate Percent Dry Wt.	Sludge Protein mg/l	Sludge Protein Percent Dry Wt.	Duration of Experiment Hrs:Min: Removal of Exogenous Substrate	Endogenous Phase	Total	Status of Endogenous Phase
						Growing System						
I	29	20.7	2280	110	20	18.2	62	56.4	11:00	923:00	934:00	Completed
II	31	8.3	2340	283	70	24.7	73	25.8	10:30	405:00	415:30	Not Completed
III	33	3.1	3430	1110	240	21.6	380	34.2	5:45	267:15	273:00	Not Completed
						Nonproliferating System						
I	29	20.5	2300	112	20	17.9	62	55.4	264:00	331:00	595:00	Not Completed
II	31	7.0	2240	318	70	22.0	88	27.7	66:30	349:00	415:30	Completed
III	33	3.1	3370	1088	225	20.7	380	34.9	10:15	262:45	273:00	Completed

TABLE XXXV

SUMMARY OF CHARACTERISTICS OF GLYCEROL SYSTEMS AT THE TIME OF REMOVAL OF EXOGENOUS SUBSTRATE

Exp. No.	Fig. No.	Time Hrs:Min	COD		Sludge			Sludge Carbohydrate					Sludge Protein				
								Total		Synthesis			Total		Synthesis		
			Residual mg/l	Removed mg/l	Total mg/l	Synthesis mg/l	Synthesis Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Sludge Synthesized	Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Sludge Synthesized	Yield Percent
								Growing System									
I	29	11:00	117	2163	1110	1000	46.2	214	19.3	194	19.4	9.0	750	67.6	688	68.8	31.8
II	31	10:30	88	2252	1650	1367	60.7	650	39.4	580	42.4	25.8	390	23.6	317	23.2	14.1
III	33	5:45	104	3326	2872	1762	53.0	1200	41.8	960	54.5	28.9	715	24.9	335	19.0	10.1
								Nonproliferating System									
I	29	264:00	57	2243	1225	1113	49.6	595	48.6	575	51.7	25.6	90	7.3	28	2.5	1.2
II	31	66:30	104	2136	935	617	28.9	-	-	-	-	-	125	13.4	37	6.0	1.7
III	33	10:15	74	3301	2652	1564	47.4	1410	53.2	1185	75.8	35.9	440	16.6	60	3.8	1.8

TABLE XXXVI

SUMMARY OF SLUDGE CHARACTERISTICS IN THE ENDOGENOUS PHASE OF GLYCEROL SYSTEMS

			Sludge					Sludge Carbohydrate					Sludge Protein				
				Final Synthesis				Final Content		Final Synthesis			Final Content		Final Synthesis		
Exp. No.	Fig. No.	Time Hrs:Min	Final Conc. mg/l	mg/l	Yield Percent	As Percent Sludge Synthesized	As Percent of Theoretical O_2 Demand	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Maximum Synthesized	Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Maximum Synthesized	Yield Percent
								Growing System									
I	29	294:30*	132	22	1.0	2.2	1.00	20	15.2	nil	-	nil	97	73.5	35	5.1	1.6
II	31	415:30	522	239	10.6	17.5	10.2	126	24.1	56	9.7	2.5	187	35.8	114	36.0	5.1
III	33	273.00	1510	400	12.0	22.7	11.7	275	18.2	35	3.6	1.1	750	49.7	370	88.1	11.1
								Nonproliferating System									
I	29	595:00	1010	898	40.0	80.7	39.0	495	49.0	475	82.6	21.2	88	8.7	26	92.9	1.2
II	31	415.30	700	382	17.9	61.9	17.0	190	27.1	120	-	0.6	100	14.3	12	32.4	0.5
III	33	273:0	1745	657	19.9	42.0	19.5	357	20.5	132	11.1	4.0	430	24.6	50	83.3	1.5

*Endogenous phase ended at this time.

Table XXXVI summarizes the effect of extended aeration on the accumulation of biological solids, carbohydrate, and protein. The data presented for the growing system of Experiment I represent the sample taken at the time of termination of the endogenous phase in the system (294 hours and 30 minutes, Figure 29). Final synthesis or net accumulation of biological solids is expressed using two terms (as percent sludge synthesized and as percent theoretical O_2 demand) which have been employed by researchers as measures of the degree of total oxidizability of biological solids.

a. Solids Accumulation Phase

It was seen in Figure 29 that the mechanism of COD removal in the growing system with low initial biological solids concentration depended upon balanced growth and replication as evidenced by the synthesis of large amounts of protein. An increase in the initial biological solids concentration (lower COD:solids ratio) caused a change in the kinetics of COD removal from logarithmic to zero order kinetics (compare Figures 29 and 33). Under nonproliferating conditions, COD removal at higher COD:solids ratios (Figures 30 and 32) was composed of a series of first-order reactions. At the lower COD:solids ratio, it followed zero-order kinetics (Figure 33). It is interesting to note that efficient substrate removal was accomplished in all nonproliferating systems, although the rate of substrate removal was considerably reduced with decrease in initial biological solids concentrations.

Solids growth was found to be exponential in all growing systems. Cell yield varied from 46 to 61 per cent (Table XXXV), and no correlation existed between cell yield and initial biological solids. Cell yields were comparable in both systems except in Experiment II, where

a low yield was obtained in the nonproliferating system. The reason for the retarded accumulation of solids in this system is not known. The appearnace of somewhat larger amounts of ribose and keto acids (Table XX) in this system would seem to suggest the relative instability of the macromolecular cell constituents (RNA and protein) or lysis of the population.

The cells in both systems preferentially synthesized carbohydrate when the initial COD:solids ratio was lowered (Table XXXV). Accumulation of carbohydrate was much higher under nonproliferating conditions. Higher initial biological solids concentration had a significant effect on the extent of carbohydrate synthesis in the nonproliferating system (Experiment III). These results suggest that efficient oxidative assimilation of glycerol could be accomplished in the absence of appreciable protein synthesis provided a low initial COD:solids ratio was employed. It is also evident that either the reversal of glycolysis from the triose phosphate stage is possible, or an alternate pathway of carbohydrate accumulation exists in these organisms.

It is doubtful whether any physical limit (saturation) was attained for the synthesis of storage compounds during these experiments. The results obtained for Experiment I (Table XXXV) show that under nonproliferating conditions where no appreciable protein synthesis took place, the cells accumulated as much as five times their weight of carbohydrate. Postgate and Hunter (163) have observed two to three-fold increases in the polysaccharide content of resting cells of Aerobacter aerogenes during metabolism of added glycerol even though the viability of the cells actually dropped by 20 to 40 per cent.

The maximum quantities of metabolic intermediates and endproducts

released into the medium by the growing and nonproliferating cells are shown in Table XXXVII.

It is seen from the table that the nonproliferating cells accumulated more intermediates or endproducts than did the growing cells; however, the difference in the relative amounts of metabolic intermediates is significant only at the highest initial COD:solids ratio. Small amounts of acetic acid were detected only in Experiment I. It has been reported that glycerol can cause partial repression of the enzymes of the TCA cycle in cells grown on minimal media (164).

Keto acids can be expected to accumulate either because of repression of the enzymes of the TCA cycle (164) or the absence of protein synthesis (69). Deamination of amino acids derived from the degradation of cell protein also leads to the formation of keto acids. In the present investigation on the metabolism of glycerol, only trace amounts of keto acids could be detected in the medium of the growing system during the metabolism of glycerol. Under nonproliferating conditions, however, somewhat higher amounts of keto acids were released during the substrate removal phase (see Tables XIX andXX). The presence of ribose along with keto acids would suggest the breakdown of protein and nucleic acid (RNA), due either to lysis or to the inability of the cells to resynthesize these macromolecules at a rate sufficient to compensate for the enhanced rate of turnover. It has been reported that RNA undergoes appreciable degradation in non-growing bacteria (165). Release of degradation products of RNA into the medium during growth of Escherichia coli in a nitrogen-deficient medium has been observed by Holme (75).

The reason for the transient accumulation of propionic acid is not

TABLE XXXVII

MAXIMUM AMOUNTS OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS RELEASED DURING METABOLISM OF GLYCEROL

Exp. No.	Fig. No.	Initial COD / Initial Biological Solids	Maximum Amounts of Intermediates and/or Endproducts	
			COD mg/l	As Percent Initial COD
		Growing System		
I	29	20.7	201	8.3
II	31	8.3	355	15.2
III	33	3.1	150	4.4
		Nonproliferating System		
I	29	20.5	410	17.8
II	31	7.0	385	17.2
III	33	3.1	197	5.8

known. It was not due, however, to lack of dissolved oxygen in the system. The DO concentration at the time propionic acid appeared in the medium was 7.4 mg/l.

b. Endogenous Phase

The endogenous oxidation or autoxidation of activated sludge is commonly expressed by the familiar law of exponential decline (166)

$$S = S_o e^{-kt}$$

where S is the biodegradable volatile solids concentration, and k is the rate of autoxidation. The fact that such an idealized situation does not exist during the entire endogenous phase can be seen from Figure 44, where a semi-log plot of solids remaining in the system versus time is shown for all sludges developed under growing conditions. It can be seen that the endogenous phase of each experiment consisted of several successive first-order reactions separated, in most cases, by a "plateau."

The values for the autoxidation rates calculated for the different phases of exponential decline of solids from the growing systems are given in Table XXXVIII. They ranged generally from 0.002 to 0.008 hr^{-1} (0.048 to 0.192 day^{-1}) except for a high rate of oxidation (0.02 hr^{-1}) observed for the initial stage of oxidation in Experiment I. The endogenous oxidation rates of activated sludge reported by workers in the waste treatment field are in the range of 0.05 to 0.285 day^{-1} (166) (167)(168). The differences in the rates of oxidation (i.e., rate of decrease in solids concentration) observed during successive phases suggest either different degrees of susceptibility of the cell components to oxidation or variations in the resistance of different

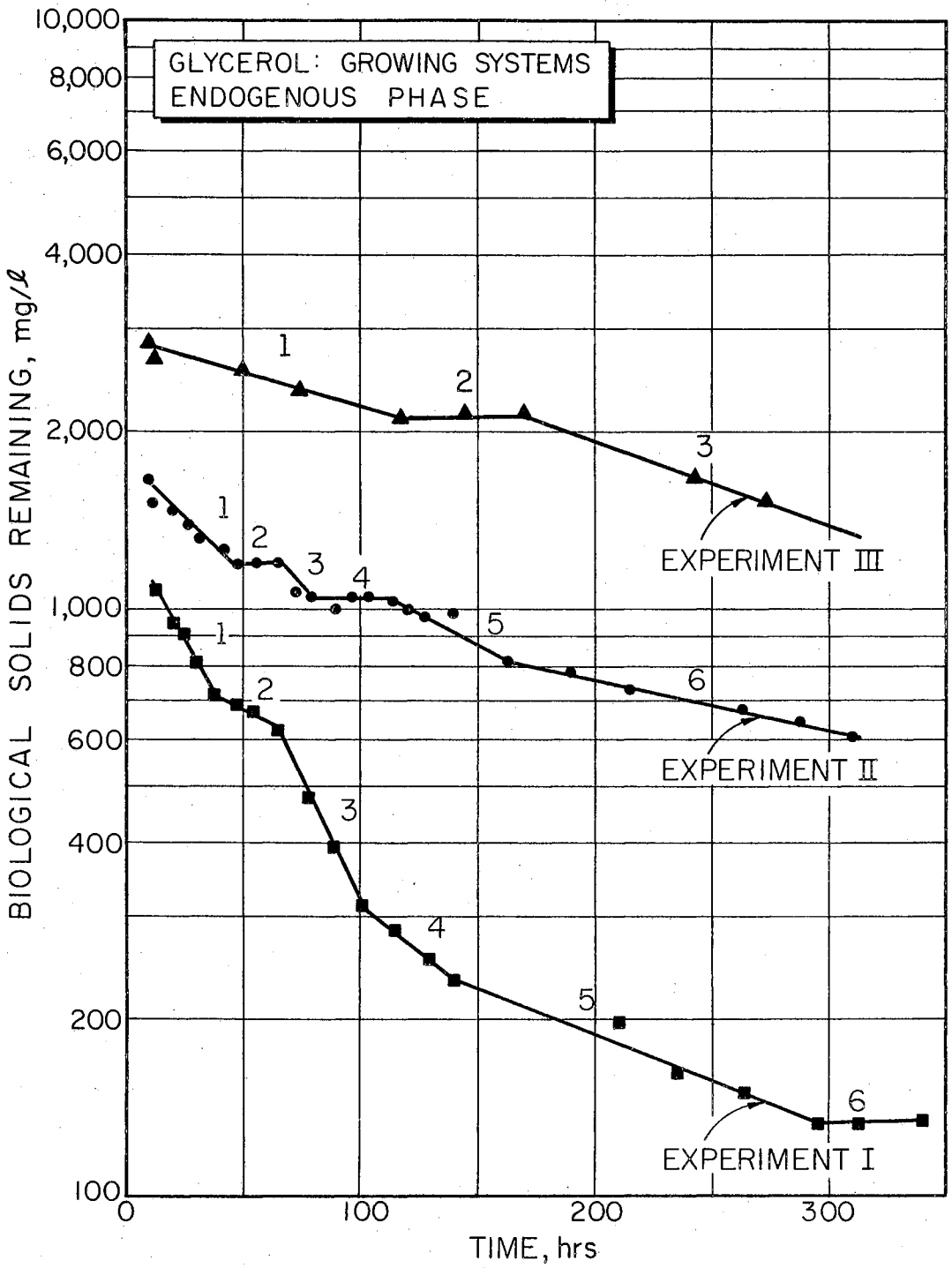


Figure 44 - Endogenous oxidation of sludges developed under growing conditions during metabolism of glycerol.

TABLE XXXVIII

ENDOGENOUS OXIDATION PHASES AND THEIR RATE CONSTANTS (k) IN SYSTEMS GROWN ON GLYCEROL

	Phase 1*		Phase 2		Phase 3		Phase 4		Phase 5		Phase 6	
Exp. No.	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}
I	19	0.0202	34	0.0062	45	0.0204	37	0.0083	156	0.0036	640	0.0000
II	38	0.0072	18	0.0000	13	-	42	0.0000	50	0.0040	252	0.0020
III	108	0.0032	52	0.0000	37	0.0030	-	-	-	-	-	-

*Number of phase corresponds to that shown in Figure 45.

populations to the adverse environment. Cells that can form spores can resist starvation conditions much longer than can others. Also, the pleteaus between oxidation phases may represent induction of specific enzymes for the degradation of certain cell components.

These endogenous oxidation curves for the sludges grown in the presence of nitrogen show a correlation between the amounts of carbohydrate accumulated by the cells during growth and the rate of oxidation of cell mass during the early endogenous phase.

It is seen that the cells in the growing system of Experiment I, which had accumulated the least amount of carbohydrate, were oxidized much more rapidly than were the cells in the other systems (compare Table XXXV and Figure 44). This indicates that the stored carbohydrate contributed to the better survival characteristics (or lower rates of endogenous oxidation) of cells in Experiments II and III (Figures 31 and 33). Also, the protein contents of the latter systems at the beginning of the endogenous phase (Table XXXV) were much smaller than the protein content of the system in Experiment I indicating, possibly, a lower number of cells to actively respire the endogenous reserve material. In the system of Experiment I (Figure 29), the greater number of cells (as assessed by the high cell protein) at the beginning of the endogenous phase, coupled with low carbohydrate reserves, fostered rapid utilization of nitrogenous cellular constituents.

The net accumulations of biological solids in both systems were shown in Table XXXVI. In order to facilitate comparison with the results reported by other workers, the values are expressed in terms which are commonly employed by researchers as measures of total oxidizability of biological solids. It is seen that the net accumulation

of solids in the growing system at the end of the endogenous phase in Experiment I was less than one per cent of the theoretical oxygen demand of the substrate fed, and two per cent of the solids synthesized during growth. This is far lower than the figures reported by Kountz and Forney (11), Washington and Symons (13), McKinney and Symons (168), and McWhorter and Heukelekian (14).

Endogenous oxidation of solids accumulated under nonproliferating conditions was considerably improved when the initial COD:biological solids ratio was reduced (Table XXXVI). However, more solids remained at the end of the endogenous metabolism of these systems than in the corresponding growth systems (compare Experiments II and III). No replication of cells would be possible in these systems during the solids accumulation phase; therefore the substrate removal as well as endogenous metabolism would be primarily dependent upon the number of cells introduced into the systems at the beginning of the experiments. Since there should be more survivors at the end of the solids accumulation phase when a larger inoculum was used, the rate of endogenous metabolism could be expected to increase with initial biological solids concentration. Also, the higher rate of survival of denser populations under starvation conditions (38)(39) might be a factor in the subsequent enhanced rate of endogenous metabolism. The endogenous oxidation in these systems, however, fell far short of complete oxidation. This would suggest loss of viability or possibly degradation of the required enzymes at the time when the solids attained a stationary level after a period of endogenous metabolism. This may also be due to the need for a prolonged acclimation or adaptation period to initiate oxidation of the remaining cell components. An alternative explanation could be the

termination of predator activity due to a lack of adequate nitrogen in their food supply.

The cell composition data presented in Table XXXV help clarify the differences in the mechanism of endogenous metabolism of solids accumulated in the presence or absence of nitrogen. Total oxidation of cellular carbohydrate occurred in the nitrogen-containing system of Experiment I during the endogenous phase. More than 90 per cent oxidation of carbohydrate was noted in the nitrogen-containing systems of Experiments II and III which were still in the active endogenous phase at the termination of the experiments. Under nonproliferating (nitrogen-starved) conditions, however, only 17 per cent of the accumulated carbohydrate was oxidized in Experiment I. More oxidation of carbohydrate occurred in the nonproliferating systems with lower initial COD:solids ratios (Experiments II and III). About 90 per cent of the carbohydrate accumulated in the absence of nitrogen was oxidized in the endogenous phase of Experiment III. Therefore, it is evident that the carbohydrate accumulated under these conditions was not non-biodegradable. These results are in agreement with Binnie, et al. (105), who observed complete endogenous utilization of carbohydrate accumulated by resting cells of Sarcina lutea.

It is also seen that the total protein level remained relatively stable throughout the endogenous phase of all nonproliferating systems, probably because of the sparing action of carbohydrate. The breakdown of protein due to either lysis or unbalanced turnover would result in the release of organic or ammonia nitrogen in the system. However, the nitrogen released by the cells could be reincorporated by the cells, since they contained a large store of carbohydrate.

The percent contents of protein and carbohydrate in the cells at the beginning and end of the experiments are also shown in Tables XXXIV and XXXVI. It can be seen that the percent carbohydrate content of the cells did not increase during the endogenous oxidation phase in growing or nonproliferating systems. These results are at variance with those reported by Symons and McKinney (10), and Washington and Symons (13), who observed a large accumulation of polysaccharide in the extended aeration process. The residual "unoxidized" carbohydrate at the end of the endogenous phase in the growing system (Experiment I) was 20 mg/l (Table XXXVI). The maximum biological solids remaining in this system at the beginning of the endogenous phase was 1125 mg/l. Thus the "nondegradable" residual (or "structural") carbohydrate represented only a meager 0.18 per cent of the total dry weight of the cells. Under nonproliferating conditions (Experiment III), the "unoxidized" carbohydrate amounted to 13 per cent of the dry weight of the cells. This latter amount probably could have been reduced further if higher initial biological solids had been employed. The "structural polysaccharide" content of microorganisms, which supposedly does not undergo detectible oxidation during endogenous metabolism has been reported to vary between 9 and 12 per cent of dry bacterial weight (105)(127).

Appreciable amounts of ribose appeared in the medium of all growing systems only after the onset of the endogenous phase. This suggests that the cellular RNA is apparently not susceptible to endogenous oxidation while exogenous substrate is present in the medium. Oxidation of ribose by the cells may have prevented accumulation of larger amounts in the medium.

The ammonia nitrogen content of the medium increased in all of the growing systems during the endogenous phase. The extent of ammonia release seems to be correlated with the type of products accumulated during the earlier growth phase. Since protein was the primary component synthesized in the growing system of Experiment I, it underwent extensive oxidation immediately upon the initiation of endogenous metabolism. Presence of appreciable amounts of ribose in the medium during this period indicates the simultaneous oxidation of RNA. Carbohydrate, which was the primary synthetic product in the growing systems of Experiments II and III, served as the immediate endogenous reserve material for the cells in these systems and delayed the oxidation of protein and release of ammonia nitrogen. Such a sparing action of carbohydrate on the endogenous oxidation of nitrogenous materials has been reported with pure cultures (124)(125)(126). A similar explanation can be offered for the absence of ammonia nitrogen in the nonproliferating system.

2. Metabolism of Sorbitol

a. Solids Accumulation Phase

As observed in earlier experiments, the kinetics and mechanism of substrate removal in sorbitol systems depended upon the initial COD: biological solids ratio. Decreasing the amount of substrate available per cell caused the substrate removal to change from logarithmic to zero-order kinetics. Similarly, COD removal kinetics changed from first-order to zero-order when the substrate concentration per cell was reduced in the absence of nitrogen. These results are essentially the same as obtained with experiments on glycerol.

The changes in measured parameters of growing and nonproliferating

systems after the removal of exogenous substrate and after different periods of endogenous metabolism are shown in Tables XXXIX, XXXX, and XXXXI. The data on sludge characteristics for the growing system of Experiment I shown in Table XXXXI correspond to the time (268 hours) when the endogenous phase was completed in the system.

There was no significant difference in cell yield under growing conditions at the three levels of COD:solids ratio employed. However, under nonproliferating conditions, cell yield was increased with decreasing COD:solids ratios. Since substrate removal in the absence of exogenous nitrogen is a function of the capacity of cells to accumulate storage materials, a larger portion of available substrate was diverted for synthesis of such compounds when a large inoculum was employed. This is evident from the higher amounts of carbohydrate synthesized by the cells at higher initial biological solids concentrations (Table XXXX).

Particular attention is drawn to the pattern of COD removal and solids growth in the nonproliferating system of Experiment I (Figure 35). Solids growth occurred only during the initial stage of COD removal. Further COD reduction did not result in an increase in biological solids. Ribose accumulation in the medium occurred during the second phase of COD removal (Table XXIII), indicating probable lysis of cells. It is interesting to compare this experiment conducted at a high initial COD:solids ratio (Experiment I, Figure 35) with the similar one with glycerol as substrate (Experiment I, Figure 29). Even though the initial COD:solids ratios in the two systems were not very much different, there was a drastic difference in substrate removal and solids accumulation characteristics between the two systems. Assuming

TABLE XXXIX

SUMMARY OF INITIAL CONDITIONS AND DURATION OF SUBSTRATE REMOVAL AND ENDOGENOUS PHASES OF SORBITOL SYSTEMS

Exp. No.	Fig. No.	Initial Conditions of System: COD / Biolog. Solids	COD mg/l	Biolog. Solids mg/l	Sludge Carbohydrate mg/l	Sludge Carbohydrate Percent Dry Wt.	Sludge Protein mg/l	Sludge Protein Percent Dry Wt.	Duration of Experiment Hrs:Min: Removal of Exogenous Substrate	Endogenous Phase	Total	Status of Endogenous Phase
						Growing System						
I	35	26.0	2290	88	23	26.1	36	40.9	10:30	933.30	944:00	Completed
II	37	14.1	2300	163	50	30.7	41	25.2	13:50	1036:10	1050:00	Not Completed
III	39	2.9	2240	775	170	21.9	185	23.9	6:30	713:30	720:00	Not Completed
						Nonproliferating System						
I	35	24.9	2320	93	26	28.0	37	39.8	-	-	944:00	Not Begun
II	37	13.3	2300	173	50	28.9	43	24.9	120:30	1097:30	1218:00	Completed
III	39	2.9	2210	775	190	24.5	190	24.5	10:45	709:15	720:00	Completed

TABLE XXXX

SUMMARY OF CHARACTERISTICS OF SORBITOL SYSTEMS AT THE TIME OF REMOVAL OF EXOGENOUS SUBSTRATE

Exp. No.	Fig. No.	Time Hrs:Min	COD		Sludge			Sludge Carbohydrate					Sludge Protein				
								Total		Synthesis			Total		Synthesis		
			Residual mg/l	Removed mg/l	Total mg/l	Synthesis mg/l	Synthesis Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Sludge Synthesized	Yield Percent	mg/l	As Percent Sludge Dry Wt.	mg/l	As Percent Sludge Synthesized	Yield Percent
								Growing System									
I	35	10:30	92	2198	1180	1092	49.7	260	22.0	237	21.7	10.8	430	36.4	394	36.1	17.9
II	37	13:50	86	2214	1400	1237	55.9	415	29.6	365	29.5	16.5	330	23.6	289	23.4	13.0
III	39	6:30	nil	2240	1992	1217	54.3	615	30.9	445	36.6	19.9	402	20.2	217	17.8	9.7
								Nonproliferating System									
I	35	944:00	390	1930	487	394	20.4	150	30.8	124	31 5	6.4	40	8.2	nil	-	-
II	37	120:30	81	2219	890	717	32.3	206	23.1	156	21.8	7.0	61	6.9	18	2.5	0.8
III	39	10:45	55	2155	1720	945	43.9	780	45.3	590	62.4	27.4	260	15.1	70	7.4	3.2

TABLE XXXXI

SUMMARY OF SLUDGE CHARACTERISTICS IN THE ENDOGENOUS PHASE OF SORBITOL SYSTEMS

Exp. No.	Fig. No.	Time Hrs:Min	Sludge: Final Synthesis: Final Conc. mg/l	Sludge: Final Synthesis: mg/l	Sludge: Final Synthesis: Yield Percent	Sludge: Final Synthesis: As Percent Sludge Synthesized	Sludge: Final Synthesis: As Percent of Theoretical O_2 Demand	Sludge Carbohydrate: Final Content: mg/l	Sludge Carbohydrate: Final Content: As Percent Sludge Dry Wt.	Sludge Carbohydrate: Final Synthesis: mg/l	Sludge Carbohydrate: Final Synthesis: As Percent Maximum Synthesized	Sludge Carbohydrate: Final Synthesis: Yield Percent	Sludge Protein: Final Content: mg/l	Sludge Protein: Final Content: As Percent Sludge Dry Wt.	Sludge Protein: Final Synthesis: mg/l	Sludge Protein: Final Synthesis: As Percent Maximum Synthesized	Sludge Protein: Final Synthesis: Yield Percent
									Growing System								
I	35	268*	272	184	8.4	16.8	8.0	50	18.4	27	11.4	1.2	100	36.8	64	16.2	2.9
II	37	1050	345	182	8.2	14.7	7.9	53	15.4	3	0.8	0.1	122	35.4	81	28.0	3.7
III	39	720	595	nil	-	-	-	162	27.2	nil	-	-	165	27.7	nil	-	-
									Nonproliferating System								
I	35	944	System did not complete substrate removal phase at this time														
II	37	1218	623	450	20.3	62.8	19.6	169	27.1	119	76.3	5.4	56	9.0	13	72.2	0.6
III	39	720	795	20	0.9	2.1	0.9	210	26.4	20	3.4	0.9	190	23.9	nil	-	-

*Endogenous phase was completed at this time.

the cells in both systems were under the influence of the same intra- and extracellular forces promoting death and lysis, the great difference in metabolic behavior between the two systems can be attributed to the greater capacity of the glycerol-grown cells to accumulate carbohydrate. Synthesis of cell carbohydrate amounted to 26 per cent of the glycerol COD removed (Table XXXV); it accounted for only about six per cent of the COD metabolized in the sorbitol system (Table XXXX). Comparison of carbohydrate accumulated in Experiments III of both sorbitol and glycerol systems in which initial COD:solids ratios were similar, also shows higher efficiency of the glycerol system to accumulate carbohydrate.

Table XXXXII shows the total amount of metabolic intermediates and endproducts released by the cells under growing and nonproliferating conditions. It can be seen from the table that nonproliferating systems produced slightly higher amounts of metabolic intermediates and endproducts. The values reported in the table also include the products (COD) released during the endogenous phase. The value reported for the nonproliferating system of Experiment I, however, represents the products of sorbitol metabolism. These products were not utilized.

Oxidation of sorbitol by bacteria has been reported to occur either at the free hexitol level with the formation of fructose (169) (170), or at the phosphorylated level with the formation of fructose-6-phosphate (171)(172)(173). Since the resorcinol reagent employed in the present study for the estimation of fructose can react with both free and esterified fructose (141), the resorcinol-positive material identified in this study can be considered to be the product of the initial step in the metabolism of sorbitol, regardless of the pathway

TABLE XXXXII

MAXIMUM AMOUNTS OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS RELEASED DURING METABOLISM OF SORBITOL

Exp. No.	Fig. No.	Initial COD / Initial Biological Solids	Maximum Amounts of Intermediates and/or Endproducts	
			COD mg/l	As Percent Initial COD
		Growing System		
I	35	26.0	248	10.8
II	37	14.1	172	7.5
III	39	2.9	203	9.0
		Nonproliferating System		
I	35	25.0	289	12.5
II	37	13.3	237	10.3
III	39	2.9	224	10.2

involved.

Presence of small amounts of acetic acid in the medium of both systems (Experiment I) indicates a similarity with glucose, sucrose, lactose, and glycerol systems. Since fructose or fructose-6-phosphate formed during the initial conversion of sorbitol can be metabolized via the glycolytic pathway, the accumulation of acetic acid can be due to the sluggishness of the TCA cycle enzyme reactions relative to the glycolytic reactions.

The data on the accumulation of carbohydrate by growing and nonproliferating cells (Table XXXX) indicate the significant influence of initial biological solids concentration on the capacity of the cells to synthesize this storage product. The probable reasons for this beneficial effect of initial solids concentration have been enumerated earlier. Synthesis of protein was the preferred reaction in systems containing nitrogen when a high COD:solids ratio was employed. With decreasing COD:solids ratio, protein synthesis lagged carbohydrate synthesis. These results were essentially similar to those obtained with glycerol systems, except in the magnitude of carbohydrate synthesis. The differences in carbohydrate yields between the two systems might have arisen due to the inhereent differences in the pathways by which these 3-carbon and 6-carbon polyhydric alcohols are metabolized.

b. Endogenous Phase

It can be seen in Figure 45 that the endogenous oxidation of solids accumulated in all growing systems was composed of at least three first-order reactions. As in the experiments with glycerol, the cells in Experiment I which accumulated the least amount of carbohydrate were oxidized much faster than those which accumulated more. The endogenous

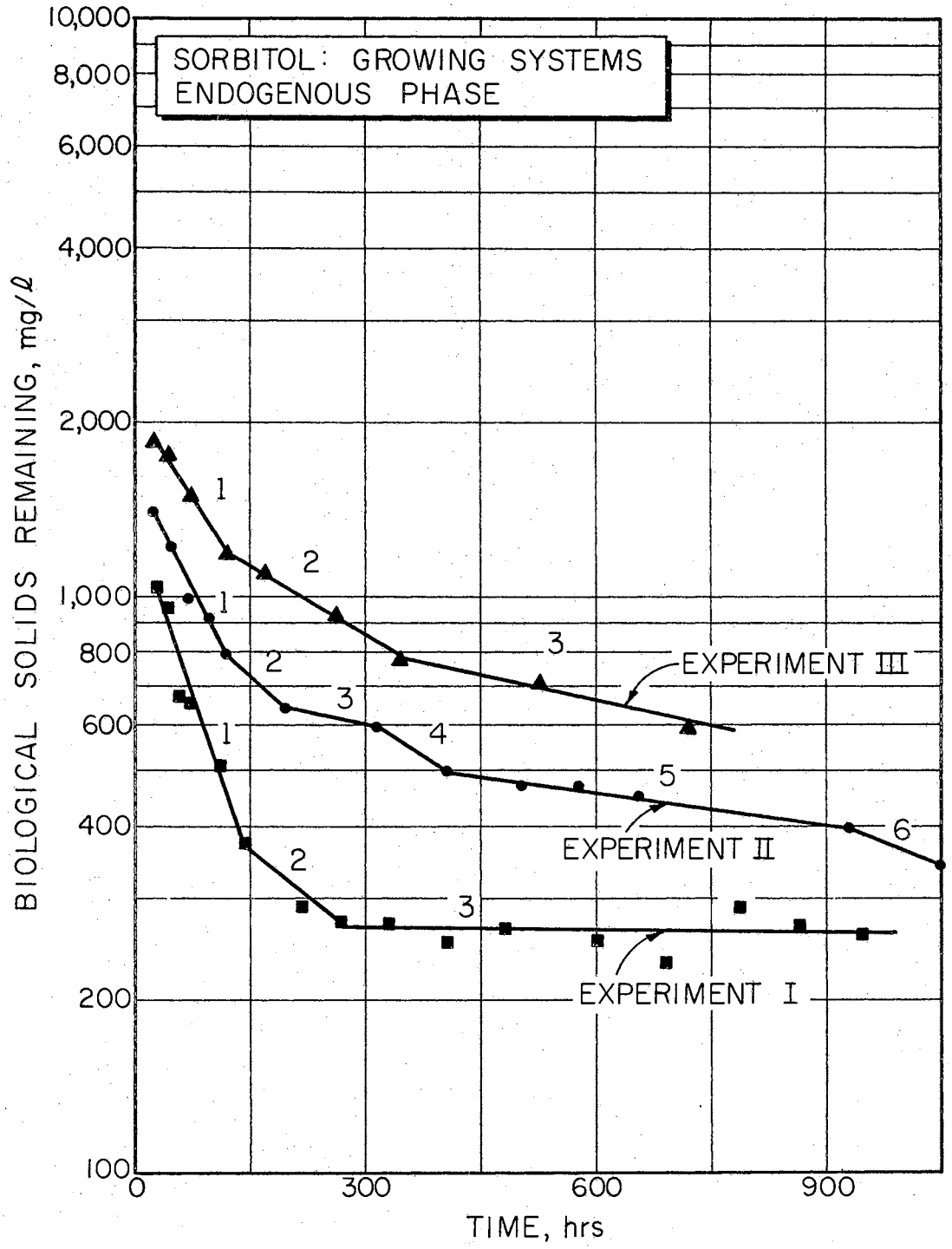


Figure 45 - Endogenous oxidation of sludges developed under growing conditions during metabolism of sorbitol.

oxidation rate constants for the initial and some succeeding phases are tabulated in Table XXXXIII. Rapid oxidation occurred only during the first 100 hours of the endogenous phase of all systems. The differences in the rates of initial oxidation between the systems are generally similar to those observed in the glycerol systems.

Examination of data summarized in Table XXXXI shows that the net accumulation of solids amounted to 17 per cent of the total solids synthesized by the growing cells in Experiment I. However, the results obtained in growing systems of Experiment II indicate somewhat higher oxidation. The solids remaining in the system at the end of the endogenous phase (Experiment I) amounted to 23 per cent of the maximum solids present in the system at the end of the solids accumulation phase. This is much larger than the 11.7 per cent observed in the glycerol system (Experiment I, Table XXXVI).

The unoxidized carbohydrate remaining in the growing system at the end of the endogenous phase in Experiment I was 50 mg/l (Table XXXXI). This represents only 4.2 per cent of the maximum solids present in the system at the end of the solids accumulation phase. In the growing system of Experiment II the corresponding figure was only 3.8 per cent. These figures indicate that the non-oxidizable "structural" carbohydrate of cells grown on sorbitol in the presence of nitrogen may not be more than four per cent of cell dry weight. Total oxidation of carbohydrate synthesized by growing systems with higher initial biological solids concentrations (Experiments II and III) occurred during the endogenous phase. Carbohydrates were preferentially utilized during the early endogenous phase of these systems. These results can be construed as evidence for the primary role of carbohydrate, accumulated by

TABLE XXXXIII

ENDOGENOUS OXIDATION PHASES AND THEIR RATE CONSTANTS (k) IN SYSTEMS GROWN ON SORBITOL

	Phase 1*		Phase 2		Phase 3		Phase 4		Phase 5		Phase 6	
Exp. No.	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}	Duration hrs	k hr^{-1}
I	114	0.0117	120	0.0026	677	0.0000	-	-	-	-	-	-
II	106	0.0051	-	-	-	-	-	-	523	0.0004	-	-
III	111	0.0047	225	0.0020	377	0.0007	-	-	-	-	-	-

*Number of phase corresponds to that shown in Figure 45.

systems with high initial biological solids concentration, as the intracellular source of carbon and energy needed for the survival and maintenance of the cells under starvation conditions.

When the synthesis of protein was predominant during the substrate removal phase (Experiment I), the pattern of endogenous metabolism was drastically altered. Protein was degraded immediately upon the entry of the cells into the endogenous phase. This finding is similar to that observed earlier in the glycerol system.

The pattern of endogenous oxidation of solids accumulated under nonproliferating conditions was somewhat different from those observed in glycerol systems. Cells grown on sorbitol in the absence of nitrogen were able to undergo more endogenous oxidation than cells grown on glycerol (compare Experiments III in Tables XXXVI and XXXXI). This was due partly to the lesser degree of oxidation of carbohydrate accumulated by the nonproliferating cells grown on glycerol.

Comparison of the cellular protein and carbohydrate contents of the growing and nonproliferating systems at the beginning and end of the experiments shows that there was no selective enrichment of the cells in both systems by carbohydrate due to endogenous metabolism, as claimed by some workers (10)(13). The protein contents of the nonproliferating cells, however, were very low, due to the lack of synthesis of protein during substrate removal and the consequent "dilution" effect.

The major endproduct released during the endogenous phase of all growing systems was ammonia nitrogen. The appearance of small amounts of ribose during the oxidation of protein in the endogenous phase suggests the similtaneous breakdown of RNA and protein. However, under

nonproliferating conditions, only RNA seemed to undergo degradation, since there was no decrease in the protein content of the systems, but ribose appeared in the medium. The absence of ammonia or organic nitrogen in the medium of these systems indicates that any nitrogen made available by the oxidation of RNA and protein was re-incorporated by the cells. The relatively larger amounts of ribose detected in the nonproliferating systems are suggestive of greater lability of RNA under these conditions.

D. General Discussion

The above discussion of the results obtained in short-term as well as long-term batch experiments was confined to the various aspects of metabolism of individual substrates. In the light of the results obtained on the oxidative assimilation of different classes of compounds, it is desirable to explore possible correlations between the kinetic and mechanistic aspects of substrate removal and solid accumulation and the prevailing environmental conditions.

1. Kinetics of COD Removal

The kinetics of solids accumulation and COD removal observed in this investigation are summarized in Table XXXXIV. The results obtained show that the kinetic behavior of substrate (COD) removal and solids growth is dependent upon the initial COD:solids ratio. At high COD:solids ratios, solids growth and COD removal followed first-order reaction kinetics with increasing rate (exponential or logarithmic growth) in the systems which were nutritionally balanced (growing systems). However, the course of COD removal in corresponding systems containing all of the nutrilites except nitrogen can be described by decreasing first-order kinetics, often having more than one phase.

TABLE XXXXIV

KINETICS OF SOLIDS ACCUMULATION AND COD REMOVAL AT DIFFERENT INITIAL COD:BIOLOGICAL SOLIDS RATIOS

Substrate	Exp. No.	Fig. No.	Initial COD / Initial Biol. Solids	Type of Kinetics: Biol. Solids Accumulation	Type of Kinetics: COD Removal	Type of Kinetics: COD Remaining
			Growing Systems			
Glucose	I	8	7.5	First Order	First Order	-
Glucose	II	10	2.8	Zero Order	-	Zero Order
Glucose	III	12	1.3	Zero Order	-	Zero Order
Sucrose	II	14	2.4	Zero Order	-	Zero Order
Sucrose	III	15	2.2	Zero Order	-	Zero Order
Acetic Acid	I	18	10.2	First Order	First Order	-
Acetic Acid	II	20	9.9	First Order	First Order	-
Acetic Acid	III	22	3.6	Zero Order	-	Zero Order
Propionic Acid	I	25	15.2	First Order	First Order	-
Propionic Acid	II	27	5.0	First Order	-	Zero Order
Glycerol	I	29	20.7	First Order	First Order	-
Glycerol	II	31	8.3	First Order	First Order	-
Glycerol	III	33	3.1	First Order	-	Mixed
Sorbitol	I	35	26.0	First Order	First Order	-
Sorbitol	II	37	14.1	First Order	First Order	-
Sorbitol	III	39	2.9	First Order	-	Mixed
			Nonproliferating Systems			
Glucose	I	8	7.5	-	-	First Order
Glucose	II	10	2.8	-	-	First Order
Glucose	III	12	1.3	Zero Order	-	Zero Order
Sucrose	II	14	2.5	-	-	First Order
Sucrose	III	15	2.4	-	-	First Order
Acetic Acid	I	18	11.4	-	-	First Order
Acetic Acid	II	20	8.6	-	-	First Order
Acetic Acid	III	22	3.3	-	-	Mixed
Propionic Acid	II	27	5.8	-	-	First Order
Glycerol	I	29	20.5	-	-	First Order
Glycerol	II	31	7.0	-	-	First Order
Glycerol	III	33	3.1	Zero Order	-	Zero Order
Sortibol	I	35	24.9	-	-	First Order
Sorbitol	II	37	13.3	-	-	First Order
Sorbitol	III	39	2.9	Zero Order	-	Zero Order

Under conditions of nitrogen starvation and consequent absence of net synthesis of enzyme (protein), the rate of substrate removal is dependent upon the number of living organisms (or total enzyme content) present initially in the systems. If the quantity of cells (or enzyme) is the only rate-limiting factor in substrate removal, then the reaction should be a linear function of time until the substrate concentration itself becomes limiting. However, since at the high COD: solids ratio the reaction rate decelerates with time, the above explanation is not consistent with the experimental results, and other factors must be considered. It seems entirely plausible that as the cells continue to store carbon, their ability to do so eventually becomes asymptotic to some upper limit. As this "saturation" limit is approached, one would expect decreasing rate kinetics. On the other hand, when the COD:solids ratio is low (i.e., high concentration of cells), the reaction rate with respect to COD removal and solids accumulation are essentially linear. In most cases the COD and solids curves approach decreasing rate kinetics during the late portions of the substrate removal period. In this case, substrate concentration becomes the rate-limiting factor, and again one would expect that a decreasing rate would be observed for both solids accumulation and COD removal. It is interesting to note that in systems operated at a low COD:solids ratio (high initial biological solids), the kinetics of solids accumulation and COD removal are similar regardless of the condition of growth, i.e., either growing or nonproliferating. With high initial solids concentration, the synthesis of storage products is the predominant metabolic reaction during the COD removal period. It has been shown by Krishnan and Gaudy (147) that even in systems containing

ammonium ion, substrate removal could be accomplished with high initial solids after complete inhibition of protein synthesis by chloramphenicol. Since the cells in both systems contained the full complement of enzymes for carbohydrate synthesis and since very little protein synthesis took place during the initial accumulation of carbohydrate even in an uninhibited growing system, there was essentially little difference in the rates of substrate removal and sludge accumulation between growing and nonproliferating systems with low initial COD:solids ratio.

2. Sludge Synthesis and Substrate Oxidation

No correlation between initial COD:solids ratio and sludge yield could be observed in the growing systems employed in the study. However, a definite relationship existed between the type of substrate and sludge yields. The highest sludge yields were obtained on carbohydrates (glucose and sucrose) followed in decreasing order by polyhydric alcohols (glycerol and sorbitol) and fatty acids.

The summary of oxygen uptake data on the carbohydrate and fatty acid systems is shown in Table XXXXV. The oxygen uptake per unit weight of substrate removed was much lower in the glucose system and somewhat lower in the sucrose system than in the systems employing acetic acid and sodium acetate as substrates. These results are in agreement with those reported by other workers in the waste treatment field (15)(94)(110)(161). The oxygen uptake data for the nonproliferating systems gave somewhat variable results. In general, higher oxidation was observed in most of the nonproliferating systems than in the accompanying growth systems. These results are at variance with those reported by others (15)(93)(94). The endogenous oxygen uptake rates ranged from 3.3 to 8.8 mg O_2/hr/gm dry solids.

TABLE XXXXV

SUMMARY OF O_2 UPTAKE DATA OF CARBOHYDRATES AND FATTY ACIDS SYSTEMS

Substrate	Exp. No.	Fig. No.	Initial COD: Theoretical or Initial COD	Initial COD: Initial Biological Solids	COD Removed mg/l	Endogenous O_2 Uptake mg/hr/gm Sludge	Total O_2 Uptake: mg/l	Total O_2 Uptake: As Percent Theoretical O_2 Demand	Total O_2 Uptake: As Percent COD Removed	Total O_2 Uptake: As mg per mg Substrate Removed
						Growing System				
Glucose	II	10	2120	2.8	1960	3.7	340	16.0	17.3	0.19
Glucose	III	12	1720	1.3	1633	4.9	231	13.4	14.1	0.15
Sucrose	I	13	1750	1.3	1610	8.6	430	24.5	26.7	0.30
Sucrose	II	14	2000	2.4	1897	-	445	22.2	23.4	0.26
Acetic Acid	II	20	950	9.9	901	8.8	325	34.2	36.0	0.39
Acetic Acid	III	22	2040	3.6	1968	4.4	550	26.9	27.9	0.30
Sodium Acetate	II	24	940	3.4	913	6.2	425	45.2	46.5	0.50
						Nonproliferating System				
Glucose	II	10	2110	2.8	1870	4.0	514	24.3	27.4	0.31
Glucose	III	12	1730	1.3	1634	4.9	238	13.8	14.6	0.16
Sucrose	I	13	1740	1.4	1275	8.7	456	26.2	35.7	0.40
Sucrose	II	14	2150	2.5	1870	-	325	15.1	17.3	0.19
Acetic Acid	II	20	925	8.6	405	7.9	250	27.0	61.7	0.66
Acetic Acid	III	22	2040	3.3	1809	3.3	630	30.8	34.8	0.37
Sodium Acetate	II	24	1000	3.1	878	4.7	495	49.5	56.3	0.60

3. Endogenous Oxidation of Sludge

The results obtained on the extended aeration of once-fed batch systems on glycerol and sorbitol help clarify somewhat conflicting concepts concerning the theoretical possibility or impossibility of total oxidation, in that they show operational conditions under which it is possible and operational conditions under which it does not seem possible that total oxidation can occur. Of particular significance in this respect is the finding that the endogenous oxidation of biological solids accumulated under growing conditions cannot be described by a single decreasing first-order kinetics constant. Instead, the reaction seems to be composed of a series of first-order reactions having different rates. Also, it was observed that the initial rate of oxidation of the sludge was dependent upon the organic loading of the system, i.e., initial COD:biological solids ratio. At higher initial loading, the cells grow exponentially during the substrate removal phase and are oxidized at a higher rate in the endogenous phase than in the system at lower initial loading. This could be attributed to the presence of a larger number of cells at the beginning of the endogenous phase and lower amount of stored materials in systems at higher loading. In the absence of large reserves of energy in the form of storage products, the cells oxidize their own vital cell materials (protein and RNA) immediately after the onset of the endogenous phase. In systems with lower initial organic loading, storage of endogenous reserves predominates during the substrate removal phase, and when the cells enter the endogenous phase the stored product (carbohydrate) is preferentially oxidized. Thus the oxidation of nitrogenous polymers is spared at

least during the initial period of endogenous respiration. The number of active cells at the beginning of the endogenous phase will also be smaller in these systems, and consequently the energy required for the survival and maintenance of metabolic functions of the organisms will be less. The oxidation of carbohydrate can meet this demand for energy during the initial period of endogenous metabolism.

In the water pollution control field there is no unanimously accepted opinion concerning the influence of operating conditions on the endogenous oxidation rate or "decay" rate of organisms. The results of the present investigation agree with the findings reported by Stewart and Ludwig (174), Stewart (175), and Middlebrooks and Garland (176) that the endogenous oxidation rate (specific organism decay rate) increases with an increase in initial substrate concentration. Middlebrooks and Garland obtained specific organism decay rates in the range of 0.0018 hr^{-1} to 0.004 hr^{-1} in model extended aeration plants which were fed raw waste water. The results obtained in this study are in agreement with the above values, except that during the initial period of endogenous oxidation much higher rates were noted in the present study (see Tables XXXVIII and XXXXIII). This could be attributed to the nature of the substrates used and also to the initial COD: biological solids ratio .

4. Release of Metabolic Intermediates and Endproducts

With all of the substrates studied, the accumulation of metabolic intermediates and endproducts was greater in nonproliferating systems than in the corresponding growth systems with similar initial COD: solids ratio. Also lower amounts of intermediary metabolites were accumulated in nonproliferating systems with lower initial COD:solids

ratios. Since the catabolism of substrate need not be coupled with the energy-requiring biosynthetic reactions of the cell, the rapid disappearance of the substrate leads to a higher intracellular concentration of the various metabolic intermediates which, in the absence of synthesis of nitrogenous macromolecules, have to be diverted to secondary metabolic pathways like synthesis of reserve carbohydrate. When lower initial solids are employed, the rate of utilization of such intermediates will be limited by the concentration of the enzyme in the system. Under such circumstances, the intracellular concentrations of the intermediates of the primary catabolic pathway of the substrate build up to high levels and this eventually leads to their secretion into the external medium. Such extracellular products may be either taken back into the cell, or utilized by other organisms. When higher initial solids are employed, the amount of primary substrate available per cell will be lower and the total amount of enzymes available for secondary biosynthetic reactions will be higher. Hence there will be less necessity to secrete the intermediary products.

It was also observed that the type of substrate influenced the extent of extracellular release of metabolic products. The results showed that metabolism of acetic acid led to the largest accumulation of metabolic intermediates. Lower initial pH did not affect this process (see Figures 18 and 20). Also, the presence or absence of exogenous nitrogen had little influence on the production of intermediates in these systems when lower initial concentrations of biological solids were employed. A comparison of these acetic acid systems and the polyhydric alcohol systems (Figures 29 and 35), where much higher initial COD:solids ratios were employed, shows the influence of the

type of substrate on the extent of release of metabolic intermediates. The other substrate which gave rise to a large amount of metabolic intermediates was glucose. The possible reasons for this phenomenon have been discussed above.

5. Utilization of Ammonia Nitrogen

The amounts of nitrogen utilized by all growing systems during the substrate removal phase are given in Table XXXXVI. Attention is drawn to the last column, wherein the net nitrogen consumption of the long-term batch systems (glycerol and sorbitol) is given. This indicates that activated sludge systems aerated for an extended period of time after the removal of exogenous substrate would require smaller amounts of ammonia nitrogen supplementation, provided the effluents containing the nitrogen released during the endogenous phase are re-utilized. It can be seen from the table that the ammonia nitrogen consumption of glucose, sucrose, glycerol, and sorbitol systems are approximately in the range of 20 units of COD removed for each unit of ammonia nitrogen consumed. Also, a slightly higher COD removal per unit of ammonia nitrogen consumed was observed in systems with high initial solids concentrations. The nitrogen consumption observed for these batch studies is about the same as that observed in the continuous flow experiments (see Table III). The nitrogen requirement of fatty acid systems was, however, very much lower. These results suggest a drastic difference between the types of organisms growing on fatty acids and on other substrates.

Mention should be made of the changes in dissolved oxygen and pH of the batch systems during the course of the experiments. All systems were maintained under aerobic conditions. Typical dissolved oxygen

TABLE XXXXVI

SUMMARY OF AMMONIA NITROGEN UTILIZATION FOR BATCH SYSTEMS

Substrate	Exp. No.	Fig. No.	Initial COD / Initial Biological Solids	COD Removed mg/l	Ammonia Nitrogen, mg/l: Consumed during Growth Phase	Ammonia Nitrogen, mg/l: Released during Endogenous Phase	COD Removed / Nitrogen Consumed	COD Removed / Net Nitrogen Consumed
Glucose	I	8	7.5	2444	150	-	16.3	-
Glucose	III	12	1.3	1663	90	-	18.1	-
Sucrose	II	14	2.4	1897	104	-	18.2	-
Sucrose	III	15	2.2	1630	121	-	13.4	-
Acetic Acid	I	18	10.2	1762	28	-	62.9	-
Acetic Acid	III	22	3.6	1968	54	-	36.4	-
Propionic Acid	I	25	15.2	3020	119	-	25.4	-
Propionic Acid	II	27	5.0	4810	133	-	36.2	-
Glycerol	I	29	20.7	2163	116	64	18.6	41.6
Glycerol	II	31	8.3	2252	124	32	18.2	23.0
Glycerol	III	33	3.1	3326	107	17	31.1	37.0
Sorbitol	I	35	26.0	2198	128	84	17.2	50.0
Sorbitol	II	37	14.1	2214	97	39	22.8	38.2
Sorbitol	III	39	2.9	2240	89	71	25.2	124.4

patterns have been plotted for the long-term experiments. None of the batch systems were limited by the availability of oxygen at any time during the experiments.

The initial pH of glucose, sucrose, sorbitol, and glycerol systems was 7.0 or 7.1. However, during growth in the presence of nitrogen, the pH dropped to 6.5 or 6.6 in all of the systems. During the endogenous phase (glycerol and sorbitol systems), the pH gradually rose and the final values were in the range of 6.8 to 7.0. Under nonproliferating conditions the initial pH of all systems was 7.1, and there was absolutely no change in this value either during the substrate removal phase or endogenous phase. These results prove conclusively that the drop in pH observed in all aerobic batch systems growing on ammonia nitrogen was due to the withdrawal of NH_4^+ ions from the medium during the growth phase. The increase in pH during the endogenous phase was due to the release of ammonia (NH_3) by the cells. The drop in pH could also be effected by the production of acid intermediates. However, since no change in pH was observed in the nonproliferating systems even during the production of acid intermediates, and since the pH dropped in growing systems even in the absence of such intermediates, these observations suggest that in batch systems with adequate buffering capacity the graudal drop in pH was solely due to the removal of NH_4^+ ion.

CHAPTER VII

SUMMARY AND CONCLUSIONS

A. Continuous Flow Experiments

In the present study, the effect of dissolved oxygen concentration on both steady state behavior of heterogeneous populations and transient response to an increase in food supply has been examined over a wide range of dissolved oxygen concentrations. Based upon this study, the following conclusions are drawn:

1. In "steady state" operations at dissolved oxygen levels ranging from 0.4 to 7.4 mg/l, such parameters as COD removal efficiency, percentage of substrate respired, respiration rate, biological solids yield, ammonia nitrogen utilization, production of extracellular metabolic intermediates and/or endproducts, and sludge protein were unaffected by oxygen tension. Also, there was in these studies only a slight tendency for sludge carbohydrate to decrease as dissolved oxygen tension was increased.

2. The transient response associated with an increase in feed concentration consisted essentially of two distinct sequential responses. The first was characterized by a metabolic or physiological reaction to the increase in food supply carried out largely by the indigenous population which existed in the system at the time of changing the feed concentration. In the initial transient, at the

lower dissolved oxygen levels, a greater amount of COD appeared in the effluent than at the highest DO level employed. The secondary response involved an ecological shift or change in predominating species in the population. Both responses can involve release of metabolic products to the medium. During the primary or metabolic response, leakage of COD and presence of metabolic intermediates (in the present instance, primarily acetic and pyruvic acids) can increase concurrently with an increase in the biological solids concentration. This phenomenon is largely due to the fact that substrate dissimilation can occur at a more rapid rate than assimilation of the carbon source by the biological solids. The secondary or ecological response may involve a more deleterious disruption of system efficiency than the primary response, and it can be expected to be characterized by a rise in effluent COD which consists largely of undissimilated (original) substrate and by a concomitant decrease in the biological solids concentration. The accumulation of metabolic products, i.e., acetic and pyruvic acids, occurred at all dissolved oxygen levels studied, and it is therefore concluded that this phenomenon is not dependent upon dissolved oxygen concentration.

B. Batch Experiments

Metabolism of glucose, sucrose, lactose, sodium acetate, acetic and propionic acids, glycerol, and sorbitol by heterogeneous microbial populations of sewage origin was studied under growing (in the presence of exogenous nitrogen) and under nonproliferating (in the absence of exogenous nitrogen) conditions at various initial loading (initial COD: initial biological solids) levels. Also, the effects of extended periods of aeration on net sludge accumulation and release of soluble

endproducts under conditions of balanced growth as well as nonproliferating conditions were investigated. All experiments were carried out under aerobic conditions. The results obtained in these closed systems lead to the following conclusions:

1. COD removal and sludge synthesis were found to be exponential at high loading conditions in growing systems. In systems under similar loading conditions but deprived of an exogenous source of nitrogen, COD removal consisted of several decreasing first-order reactions. Increasing the initial biological solids level (or lowering the initial COD:solids ratio) caused the removal and solids accumulation to be linear functions of time, irrespective of the presence or absence of exogenous nitrogen.

Sludge yield in growing systems did not depend upon the initial COD:solids ratios in the range studied. However, under nonproliferating conditions, sludge yield increased with decreasing initial COD:solids ratio.

Absence of a nitrogen source in the medium and lower initial COD: solids ratios generally favored increased accumulation of cellular carbohydrate. Highest conversion of substrate COD into cell carbohydrate was noted for glucose and sucrose systems followed, in decreasing order, by glycerol, sorbitol, and fatty acids systems.

3. The extent of elaboration of metabolic intermediates and/or endproducts was primarily dependent upon the type of substrate. Largest conversion of original substrate into intermediary products was observed in acetic acid systems. Glucose ranked second in this regard. Rapid extracellular accumulation of glucose and fructose occurred during the metabolism of sucrose in the presence or absence of exogenous

nitrogen. Lactose was hydrolyzed intracellularly.

Release of extracellular metabolic products did not depend upon the initial loading conditions (initial COD:solids ratio) in a nutritionally-balanced activated sludge. However, in a system containing all nutrients in excess, but deprived of an exogenous source of nitrogen, the amount of intermediates accumulated in the medium was proportional to the initial COD:solids ratio. At the same loading levels, the nonproliferating systems produced more extracellular products than did the corresponding system supplied with nitrogen.

4. Acetic acid is an extracellular product during the metabolism of glucose, sucrose, glycerol, and sorbitol in the presence or absence of exogenous nitrogen. Pyruvic acid was identified in the medium of glucose systems. Small amounts of keto acids were detected in the medium of growing and nonproliferating systems during the metabolism of glucose, lactose, acetic acid, sorbitol, and glycerol. Transient accumulation of propionic acid was observed during the metabolism of the polyhydric alcohols. Fructose was identified in the medium of sorbitol systems. Anthrone-reactive materials were released during the metabolism of propionic acid in the absence of nitrogen.

5. Ribose appeared in the medium during the endogenous phase of growing and nonproliferating systems, suggesting the breakdown of cellular RNA due to endogenous oxidation or cell lysis. It was also released during the substrate removal phase of nonproliferating systems with a high initial COD:biological solids ratio.

Release of ammonia nitrogen into the medium was noted during the prolonged endogenous phase of all growing systems. Ammonia nitrogen was released immediately upon the onset of the endogenous phase in

systems operated at high initial COD:solids ratios. In systems operated at low initial COD:solids ratios, endogenous oxidation of carbohydrate delayed the release of ammonia nitrogen. No organic nitrogen could be detected in the medium during either the substrate removal phase or the endogenous phase of growing or nonproliferating systems.

The COD of the medium did not increase during the endogenous oxidation of the sludge accumulated during balanced growth or in the absence of nitrogen.

6. "Total oxidation" of sludge synthesized under balanced growth conditions did occur during extended periods of aeration in the absence of a carbon and energy source. The residual mass remaining at the end of the endogenous phase amounted to 2 to 15 per cent of solids synthesized in the system during the substrate removal phase. Endogenous oxidation of solids accumulated under growth conditions occurred as a series of first-order reactions, the reaction rates generally declining with time. Very high rates of oxidation were observed during the initial period of endogenous metabolism. The initial rates were dependent upon the initial loading conditions of the system. The possibility of total oxidation of cellular carbohydrate and protein synthesized during the growth phase was demonstrated in this study. No accumulation of biologically non-oxidizable carbohydrate was observed.

The rate of endogenous oxidation was much slower for sludges accumulated in the absence of nitrogen than for sludge of high protein content, and a large part of the sludge remained unoxidized at the end of the endogenous phase. Increased rates of oxidation were noted for systems which received lower initial loadings. It was demonstrated that cellular carbohydrate accumulated in the absence of exogenous

nitrogen was susceptible to almost total endogenous oxidation, provided the initial COD:solids ratio was low.

CHAPTER VIII

SUGGESTIONS FOR FUTURE WORK

1. Further studies on the metabolic response of activated sludge to dissolved oxygen tensions in a range lower than that employed in the present investigation should be conducted.

2. The generality of the occurrence of the "secondary response" should be fully explored, using mixed cultures of organisms known to be prevalent in activated sludge. It would be interesting to study the changes in ecology of the steady state populations due to the introduction of known metabolic products (i.e., acetic and pyruvic acids when glucose is used as the carbon source), along with the substrate in the feed. Also, the effect of increasing the free acid concentrations (lowering the pH) in such feed should be studied.

3. Attempts should be made to identify and quantitate other metabolic intermediates and/or endproducts released during the aerobic metabolism of substrates like glucose and acetic acid.

4. The effect of phosphorus deficiency on the release of metabolic intermediates and endproducts should be investigated.

5. Additional work is needed concerning the effect of the nature of the original substrate and initial COD:biological solids ratio on the rate of autodigestion of sludges in the endogenous phase.

6. More information on the ecology of the activated sludge during

the prolonged endogenous phase is essential in order to interpret the kinetic behavior of sludge oxidation observed in the present study. The possibility of accelerating the endogenous oxidation of sludges accumulated in the absence of nitrogen by addition of a source of nitrogen at the time of termination of the solids accumulation phase should be investigated.

7. Since total oxidation of the sludge mass synthesized during the substrate removal phase occurred in the endogenous phase, it would be worthwhile to investigate whether the residual sludge mass is capable of repeating the sludge accumulation and total oxidation cycle.

SELECTED BIBLIOGRAPHY

1. Committee on Pollution, National Academy of Sciences, National Research Council, Waste Management and Control, Publication 1400, National Academy of Sciences, National Research Council, Washington, D. C. (1966).

2. Gloyna, E., Wolff, J., Geyer, J., and Wolman, A., A Report Upon the Present and Prospective Means for Improved Reuse of Water, 1-54, Committee Print No. 30. Select Committee on National Water Resources, U. S. Senate. United States Government Printing Office, Washington, D. C. (1960).

3. Advanced Waste Treatment Branch, Division of Research, Robert A. Taft Sanitary Engineering Center, Summary Report, Advanced Waste Treatment Research Program, July 1965-July 1967, U. S. Department of Interior, Federal Water Pollution Control Administration, Cincinnati, Ohio (1968).

4. Ettinger, M. B., "Symposium on Joint versus Separate Treatment of Municipal and Industrial Wastes." Journal Water Pollution Control Federation, 36, 345-361 (1964).

5. RamaRao, C. V., Speece, R. E., and Engelbrecht, R. S., "The Response of Activated Sludge to Nitrogen-deficient Conditions." Journal Water Pollution Control Federation, 37, 1422-1436 (1965).

6. McKinney, R. E., and O'Brien, W. J., "Activated Sludge--Basic Design Concepts." Journal Water Pollution Control Federation, 40, 1831-1843 (1968).

7. Komolrit, K., Goel, K. C., and Gaudy, A. F., Jr., "Regulation of Exogenous Nitrogen Supply and its Possible Applications to the Activated Sludge Process." Journal Water Pollution Control Federation, 39, 251-266 (1967).

8. Gaudy, A. F., Jr., Goel, K. C., and Gaudy, E. T., "Continuous Oxidative Assimilation of Acetic Acid and Endogenous Protein Synthesis Applicable to Treatment of Nitrogen-deficient Waste Waters." Applied Microbiology, 16, 1358-1363 (1968).

9. Gaudy, A. F., Jr., Goel, K. C., and Freedman, A. J., "Activated Sludge Process Modification for Nitrogen-deficient Wastes." Paper prepared for Proceedings, 4th International Conference on Water Pollution Control Research, Prague, Czechoslovakia, September, 1968, in press.

10. Symons, J. M., and McKinney, R. E., "The Biochemistry of Nitrogen in the Synthesis of Activated Sludge." Sewage and Industrial Wastes, 30, 874-890 (1958).

11. Kountz, R. R., and Forney, C., Jr., "Metabolic Energy Balances in a Total Oxidation Activated Sludge System." Sewage and Industrial Wastes, 31, 819-826 (1959).

12. Busch, A. W., and Myrick, N., "Food-Population Equilibria in Bench-scale Bio-oxidation Units." Journal Water Pollution Control Federation, 32, 949-959 (1960).

13. Washington, D. R., and Symons, J. M., "Volatile Sludge Accumulation in Activated Sludge Systems." Journal Water Pollution Control Federation, 34, 767-790 (1962).

14. McWhorter, T. R., and Heukelekian, H., "Growth and Endogenous Phases in the Oxidation of Glucose." In Advances in Water Pollution Research, 2, 419-449, edited by W. W. Eckenfelder. The Macmillan Company, New York (1964).

15. Gaudy, A. F., Jr., and Engelbrecht, R. S., "Basic Biochemical Considerations During Metabolism in Growing versus Respiring Systems." In Advances in Biological Waste Treatment, 11-16, edited by W. W. Eckenfelder, Jr., and J. McCabe. Pergamon Press, London (1963).

16. Gaudy, A. F., Jr., and Engelbrecht, R. S., "Quantitative and Qualitative Shock Loading of Activated Sludge Systems." Journal Water Pollution Control Federation, 33, 800-816 (1961).

17. Krishnan, P., and Gaudy, A. F., Jr. Unpublished results.

18. Rickard, M. D., "The Effect of Agitation on the Behavior of Completely Mixed Bacterial Systems." Ph.D. Thesis, Oklahoma State University (1968).

19. Murtaugh, J. J., and Bunch, R. L., "Acidic Components of Sewage Effluents and River Water." Journal Water Pollution Control Federation, 37, 410-415 (1965).

20. Lamanna, C., and Mallette, M. F., Basic Bacteriology and its Biological and Chemical Background. The Williams and Wilkins Company, Baltimore (1959).

21 Sawyer, C. N., "Bacterial Nutrition and Synthesis." In Biological Treatment of Sewage and Industrial Wastes. I. Aerobic Oxidation, 3-17, edited by J. McCabe and W. W. Eckenfelder, Jr. Reinhold Publishing Corporation, New York (1956).

22. Senez, J. C., "Some Considerations on the Energetics of Bacterial Growth." Bacteriological Reviews, 26, 95-107 (1962).

23. Gunsalus, I. C., and Shuster, C. W., "Energy-yielding Metabolism in Bacteria." In The Bacteria, Volume II, edited by I. C. Gunsalus and R. Y. Stanier. Academic Press, Inc., New York (1961).

24. Forrest, W. W., and Walker, D. J., "Control of Glycolysis in Washed Suspensions of Streptococcus faecalis." Nature, 207, 46-48 (1965).

25. Madsen, N. B., "The Biological Control of Glycogen Metabolism in Agrobacterium tumefaciens." Canadian Journal of Biochemistry and Physiology, 41, 561-571 (1963).

26. Preiss, J., Shen, L., Greenberg, E., and Gentner, N., "Biosynthesis of Bacterial Glycogen. IV. Activation and Inhibition of the Adenosine Diphosphate Glucose Pyrophosphorylase of Escherichia coli B." Biochemistry, 5, 1833-1845 (1966).

27. Lamanna, C., "Studies of Endogenous Metabolism in Bacteriology." Annals New York Academy of Sciences, 102, 517-520 (1963).

28. Dawes, E. A., and Ribbons, D. W., "Studies on the Endogenous Metabolism of Escherichia coli." Biochemical Journal, 95, 332-343 (1965).

29. McGrew, S. B., and Mallette, M. F., "Energy of Maintenance in Escherichia coli." Journal of Bacteriology, 83, 844-850 (1962).

30. Marr, A. G., Nilson, E. J., and Clark, D. J., "The Maintenance Requirement of Escherichia coli." Annals New York Academy of Sciences, 102, 536-548 (1963).

31. Strange, R. E., Wade, H. E., and Dark, F. A., "Effect of Starvation on Adenosine Triphosphate Concentration in Aerobacter aerogenes." Nature, 199, 55-57 (1963).

32. Welsch, M., "Lysis by Agents of Microbial Origin." Journal of General Microbiology, 18, 491-497 (1958).

33. Hadjipetrou, L. P., and Stouthamer, A. H., "Autolysis of Bacillus subtilis by Glucose Depletion." Antonie van Leeuwenhoek Journal of Microbiology and Serology, 29, 256-260 (1963).

34. Sierra, G., and Zagt, R., "Some Remarks on Autolysis of Pseudomonas aeruginosa." Antonie van Leeuwenhoek Journal of Microbiology and Serology, 26, 193-208 (1960).

35. Ryan, F. J., "Bacterial Mutation in a Stationary Phase and the Question of Cell Turnover." Journal of General Microbiology, 21, 530-549 (1959).

36. Postgate, J. R., and Hunter, J. R., "The Survival of Starved Bacteria." Journal of General Microbiology, 29, 233-263 (1962).

37. Postgate, J. R., "Viability Measurements and the Survival of Microbes Under Minimum Stress." In Advances in Microbial Physiology, 1, 1-21 (1967).

38. Harrison, A. P., Jr., "The Response of Bacterium lactis aerogenes when Held at Growth Temperature in the Absence of Nutriment : An Analysis of Survival Curves." Proceedings Royal Society (London), (B)152, 418-428 (1960).

39. Postgate, J. R., and Hunter, J. R., "The Survival of Starved Bacteria." Journal of Applied Bacteriology, 26, 295-306 (1963).

40. Carter, B. L. A., and Bull, A. T., "Glucose Metabolism in Aspergillus nidulans." Journal of General Microbiology, 53, x (1968).

41. Ecker, R. E., and Lockhart, W. R., "Specific Effect of Limiting Nutrient on Physiological Events During Culture Growth." Journal of Bacteriology, 82, 511-516 (1961).

42. Pirt, S. J., "The Oxygen Requirement of Growing Cultures of an Aerobacter Species Determined by Means of the Continuous Culture Technique. Journal of General Microbiology, 16, 59-75 (1957).

43. Moss, F., "The Influence of Oxygen Tension on Respiration and Cytochrome a_2 Formation of Escherichia coli. The Australian Journal of Experimental Biology and Medical Science, 30, 531-540 (1952).

44. Herbert, D., "Microbial Respiration and Oxygen Tension." Journal of General Microbiology, 41, viii-ix (1965).

45. Lockhart, W. R., "Use of Microorganisms for Studies of Growth and Morphogenesis." Bacteriological Reviews, 23, 8-17 (1959).

46. Winzler, R. J., "The Respiration of Bakers' Yeast at Low Oxygen Tension." Journal of Cellular and Comparative Physiology, 17, 263-276 (1941).

47. Longmuir, I. S., "Respiration Rate of Bacteria as a Function of Oxygen Concentration." Biochemical Journal, 57, 81-87 (1954).

48. Johnson, M. J., "Aerobic Microbial Growth at Low Oxygen Concentrations." Journal of Bacteriology, 94, 101-108 (1967).

49. Tsao, G. T. N., and Kempe, L. L., "Oxygen Transfer in Fermentation Systems. I. Use of Gluconic Acid Fermentation for Determination of Instantaneous Oxygen Transfer Rates." Journal Biochemical and Microbiological Technology Engineering, 2, 129-142 (1960).

50. Rickard, M. D., and Gaudy, A. F., Jr., "Effect of Mixing Energy on Sludge Yield and Cell Composition for Heterogeneous Populations in Completely Mixed Systems." Journal Water Pollution Control Federation, Research Supplement, 40, R129-144 (1968).

51. Herbert, D., "Some Principles of Continuous Culture." Recent Progress in Microbiology, Symposia, VII. International Congress for Microbiology (Stockholm) 1958, 381-396. Almquist and Wicksell, Stockholm, Sweden (1959).

52. Gaden, E. L., Jr., "Aeration and Oxygen Transport in Biological Systems--Basic Considerations." In Biological Treatment of Sewage and Industrial Wastes. I. Aerobic Oxidation, 172-191, edited by J. McCabe and W. W. Eckenfelder, Jr. Reinhold Publishing Corporation, New York (1956).

53. Davis, B. D., "Teleonomic Significance of Biosynthetic Control Mechanisms." Cold Spring Harbor Symposia on Quantitative Biology, 26, 1-10 (1961).

54. Nakata, H. M., and Halvorson, H. O., "Biochemical Changes Occurring During Growth and Sporulation of Bacillus cereus." Journal of Bacteriology, 80, 801-810 (1960).

55. Hansen, R. S., Srinivasan, V. R., and Halvorson, H. O., "Biochemistry of Sporulation. I. Metabolism of Acetate by Vegetative and Sporulating Cells." Journal of Bacteriology, 85, 451-460 (1963).

56. Szulmajster, J., and Hansen, R. S., "Physiological Control of Sporulation in Bacillus subtilis." In Spores, III, 162-173, edited by H. O. Halvorson and L. L. Campbell. American Society for Microbiology, Ann Arbor, Michigan (1965).

57. Bhatla, M. N., and Gaudy, A. F., Jr., "Studies on the Plateau in Oxygen Uptake During Exertion of Biochemical Oxygen Demand by Pure Cultures of Bacteria." Biotechnology and Bioengineering VII, 387-404 (1965).

58. Neidhardt, F. C., and Magasanik, B., "Inhibitory Effect of Glucose on Enzyme Formation." Nature, 178, 801-802 (1956).

59. Neidhardt, F. C., "Mutant of Aerobacter aerogenes Lacking Glucose Repression." Journal of Bacteriology, 80, 536-543 (1960).

60. Mandelstam, J., "Repression of Constitutive β-Galactosidase in Escherichia coli by Glucose and Other Carbon Sources." Biochemical Journal, 82, 489-493 (1962).

61. Forget, P., and Pichinoty, F., Le Cycle Tricarboxylique chez Aerobacter aerogenes." Annales de L'Institut Pasteur, 112, 261-290 (1967).

62. Gray, C. T., Wimpenny, J. W. T., and Mossman, M. R., "Regulation of Metabolism in Facultative Bacteria. II. Effects of Aerobiosis, Anaerobiosis, and Nutrition on the Formation of Krebs Cycle Enzymes in Escherichia coli." Biochimica et Biophysica Acta, 117, 33-41 (1966).

63. Amarasingham, C. R., and Davis, B. D., "Regulation of α-Ketoglutarate Dehydrogenase in Escherichia coli." Journal of Biological Chemistry, 240, 3664-3668 (1965).

64. Polakis, E. S., and Bartley, W., "Changes in Enzyme Activities of Saccharomyces cerevisiae During Aerobic Growth on Different Carbon Sources." Biochemical Journal, 97, 284-297 (1965).

65. Ivler, D., "Comparative Metabolism of Virulent and Avirulent Staphylococci." Annals New York Academy of Sciences, 128, 62-80 (1965).

66. Polakis, E. S., and Bartley, W., "Changes in the Intracellular Concentrations of Adenosine Phosphates and Nicotinamide Nucleotides During Aerobic Growth Cycle of Yeast on Different Carbon Sources." Biochemical Journal, 99, 521-533 (1966).

67. Eagon, R. G., and Cho, H. W., "Major Products of Glucose Dissimilation by Pseudomonas natrigens." Journal of Bacteriology, 89, 1209-1211 (1965).

68. Caputto, R., Barra, H. S., and Cumar, F. A., "Carbohydrate Metabolism." Annual Review of Biochemistry, 36, 211-246 (1967).

69. Duncan, M. G., and Campbell, J. J. R., "Oxidative Assimilation of Glucose by Pseudomonas aeruginosa." Journal of Bacteriology, 84, 784-792 (1962).

70. Allen, S. H. G., Jr., and Powelson, O., "Pathways of Glucose Oxidation in Dividing and Non-dividing Cells of Escherichia coli." Journal of Bacteriology, 75, 184-189 (1958).

71. Moss, F., "Adaptation of Cytochromes of Aerobacter aerogenes in Response to Environmental Oxygen Tension." The Australian Journal of Experimental Biology and Medical Science, 34, 395-405 (1956).

72. Englesberg, E., Levy, J. B., and Gibor, A., "Some Enzymatic Changes Accompanying the Shift From Anaerobiosis in Pasteurella pestis." Journal of Bacteriology, 68, 178-185 (1954).

73. Clifton, C. E., "Influence of Growth Medium on Assimilatory Activities of Escherichia coli." Journal of Bacteriology, 85, 1371-1377 (1963).

74. Monod, J., "The Growth of Bacterial Cultures." Annual Reviews of Microbiology, 3, 371-394 (1949).

75. Holme, T., "Continuous Culture Studies on Glycogen Synthesis in Escherichia coli B." Acta Chemica Scandinavica, 11, 763-775 (1957).

76. Storer, F., Jr., "Some Kinetic Properties of Transient States in Continuous Cultures of Natural Microbial Populations Induced by Increasing the Feed Concentration of the Growth-limiting Nutrient." MS Thesis, Oklahoma State University (1968).

77. Brock, T. D., Principles of Microbial Ecology. Prentice Hall, Inc., New Jersey (1966).

78. Stotzky, G., and Norman, A. G., "Factors Limiting Microbial Activities in Soil. I. The Level of Substrate, Nitrogen, and Phosphorus." Archiv für Mikrobiologie, 40, 341-369 (1961).

79. Bungay, H. R., III, and Bungay, M. L., "Microbial Interactions in Continuous Culture." In Advances in Applied Microbiology, 10, 269-290 (1968).

80. Cassell, E. A., Sulzer, F. T., and Lamb, J. C., III, "Population Dynamics and Selection in Continuous Mixed Cultures." Journal Water Pollution Control Federation, 38, 1398-1409 (1966).

81. Chian, S. K., and Mateles, R. I., "Growth of Mixed Cultures on Mixed Substrates. I. Continuous Culture." Applied Microbiology, 16, 1337-1342 (1968).

82. Bustamante, R. B., "Studies on Bacterial Predominance Patterns in Mixed Cultures." Ph.D. Thesis, Oklahoma State University (1968).

83. Shindala, A., Bungay, H. R., III, Kreig, N. R., and Culbert, K., "Mixdd Culture Interactions. I. Commensalism of Proteus vulgaris with Saccharomyces cerevisiae in Continuous Culture." Journal of Bacteriology, 89, 693-696 (1965).

84. Okun, D. A., and Lynn, W. R., "Preliminary Investigations Into the Effect of Oxygen Tension on Biological Sewage Treatment." In Biological Treatment of Sewage and Industrial Wastes. I. Aerobic Oxidation, 192-207, edited by J. McCabe and W. W. Eckenfelder, Jr. Reinhold Publishing Corporation, New York (1956).

85. Smith, D. B., "Aerobic Biological Stabilization of Organic Substrates." Sewage and Industrial Wastes, 24, 1077-1090 (1952).

86. Gaudy, A. F., Jr., and Turner, B. G., "Effect of Airflow Rate on Response of Activated Sludge to Quantitative Shock Loading." Journal Water Pollution Control Federation, 36, 767-781 (1964).

87. Baumberger, J. P., "The Relation Between 'Oxidation-Reduction Potential' and the Oxygen Consumption Rate of Yeast Cell Suspensions." Cold Spring Harbor Symposia on Quantitative Biology, 7, 195-215 (1939).

88. Phillips, D. H., and Johnson, M. J., "Aeration in Fermentation." Journal of Biochemical and Microbiological Technology and Engineering, 3, 277-309 (1961).

89. Harrison, D. E. F., and Pirt, S. J., "The Influence of Dissolved Oxygen Concentration on the Respiration and Glucose Metabolism of Klebsiella aerogenes During Growth." Journal of General Microbiology, 46, 193-211 (1967).

90. Smith, C. G., and Johnson, M. J., "Aeration Requirements for the Growth of Aerobic Microorganisms." Journal of Bacteriology, 68, 345-350 (1954).

91. Porges, N., Jasewicz, L., and Hoover, S. R., "A Microbiological Process Report: Aerobic Treatment of Dairy Wastes." Applied Microbiology, 1, 262-270 (1953).

92. von der Emde, W., "Aspects of the High Rate Activated Sludge Process." In Advances in Biological Waste Treatment, 299-317, edited by W. W. Eckenfelder, Jr., and J. McCabe. Pergamon Press, New York (1963).

93. Wuhrmann, K., "Factors Affecting Efficiency and Solids Production in Activated Sludge Process." In Biological Treatment of Sewage and Industrial Wastes. I. Aerobic Oxidation, 49-65, edited by J. McCabe and W. W. Eckenfelder, Jr. Reinhold Publishing Corporation, New York (1956).

94. van Gils, H. W., "Bacteriology of Activated Sludge." Report No. 32, Research Institute for Public Health Engineering, The Hague, Holland (July, 1964).

95. Rickard, M. D., and Gaudy, A. F., Jr., "Effect of Oxygen Tension on O_2 Uptake and Sludge Yield in Completely Mixed Heterogeneous Populations." Proceedings 23rd Industrial Waste Conference, Purdue University, Lafayette, Indiana (1968)(in press).

96. Wuhrmann, K., "Effect of Oxygen Tension on Biochemical Reactions in Sewage Purification Plants." In Advances in Biological Waste Treatment, 27-40, edited by W. W. Eckenfelder, Jr., and J. McCabe. Pergamon Press, New York (1963).

97. Orford, H. E., Heukelekian, H., and Isenberg, E., "Effect of Sludge Loading and Dissolved Oxygen on the Performance of the Activated Sludge Process." In Advances in Biological Waste Treatment, 251-263, edited by W. W. Eckenfelder, Jr., and J. McCabe. Pergamon Press, New York (1963).

98. Heukelekian, H., "Oxygen Tension and Bacterial Numbers." Sewage Works Journal, 8, 415-421 (1936).

99. Okun, D. A., "System of Bio-Precipitation of Organic Matter From Sewage." Sewage Works Journal, 21, 763-794 (1949).

100. Barker, H. A., "The Oxidative Metabolism of the Colorless Alga, Prototheca zopfii." Journal of Cellular and Comparative Physiology, 8, 231-250 (1936).

101. Clifton, C. E., "Microbial Assimilations." Advances in Enzymology, 6, 269-308 (1946).

102. Clifton, C. E., "Endogenous Metabolism and Oxidative Assimilation of Typical Bacterial Species." Annals New York Academy of Sciences, 102, 655-668 (1963).

103. Clifton, C. E., and Sobek, J. M., "Oxidative Assimilation of Glucose and Endogenous Respiration of Bacillus cereus." Journal of Bacteriology, 81, 284-287 (1961).

104. Clifton, C. E., "Oxidative Assimilation and Distribution of Glucose in Bacillus cereus." Journal of Bacteriology, 83, 66-69 (1962).

105. Binnie, B., Dawes, E. A., and Holms, W. H., "Metabolism of Sarcina lutea. IV. Patterns of Oxidative Assimilation." Biochimica et Biophysica Acta, 40, 237-251 (1960).

106. Clifton, C. E., "Oxidative Assimilation by Bacillus megaterium." Journal of Bacteriology, 85, 1365-1370 (1963).

107. Macrae, R. M., and Wilkinson, J. F., "Poly-β-hydroxybutyrate Metabolism in Washed Suspensions of Bacillus cereus and Bacillus megaterium." Journal of General Microbiology, 19, 210-222 (1958).

108. Gronlund, A. F., and Campbell, J. J. R., "Nitrogenous Compounds as Substrates for Endogenous Respiration in Microorganisms." Journal of Bacteriology, 81, 721-724 (1961).

109. Ruchhoft, C. C., Kachmar, J. F., and Placak, O. R., "Studies of Sewage Purification. XII. Metabolism of Glucose by Activated Sludge." Sewage Works Journal, 12, 485-503 (1940).

110. Placak, O. R., and Ruchhoft, C. C., "Studies of Sewage Purification. XVII. The Utilization of Organic Substrates by Activated Sludge." Sewage Works Journal, 19, 423-440 (1947).

111. Porges, N., Jasewicz, L., and Hoover, S. R., "Biochemical Oxidation of Dairy Wastes. VII. Purification, Oxidation, Synthesis, and Storage." Proceedings 10th Industrial Waste Conference, Purdue University, Lafayette, Indiana, Ext. Ser. 89, 135-146 (1955).

112. Pannbacker, R. G., and Wright, B. E., "Carbohydrate Accumulation in the Protist - Biochemical Model of Differentiation." In Chemical Zoology, 573-616, edited by M. Florkin and B. T. Scheer. Academic Press, New York (1967).

113. Dagley, S., and Johnson, A. R., "The Relation Between Lipid and Polysaccharide Contents of Bacterium coli." Biochimica et Biophysica Acta, 11, 158-159 (1953).

114. Walters, C. F., Engelbrecht, R. S., and Speece, R. E., "Microbial Substrate Storage in Activated Sludge." Journal Sanitary Engineering Division, Proceedings ASCE, 94, 257-269 (1968).

115. Goel, K. C., and Gaudy, A. F., Jr., "Regeneration of Oxidative Assimilation Capacity by Intracellular Conversion of Storage Products to Protein." Applied Microbiology, 16, 1352-1357 (1968).

116. Adamse, A. D., Bacteriological Studies on Dairy Waste Activated Sludge. H. Veenman En Zonen N. V. - Wageningen (1966).

117. MacKelvie, R. M., Campbell, J. J. R., and Gronlund, A. F., "Absence of Storage Products in Cultures of Pseudomonas aeruginosa Grown With Excess Carbon or Nitrogen." Canadian Journal of Microbiology, 14, 627-631 (1968).

118. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., and Britten, R. J., "Studies on Biosynthesis in Escherichia coli." Carnegie Institution of Washington Publication 607, Washington, D. C. (1963).

119. Hadjipetrou, L. P., Gerrits, J. P., Teulings, F. A. G., and Stouthamer, A. H., "Relation Between Energy Production and Growth of Aerobacter aerogenes." Journal of General Microbiology, 36, 139-150 (1964).

120. George, T. K., and Gaudy, A F., Jr., Unpublished results.

121. Komolrit, K., and Gaudy, A. F., Jr., "Biochemical Response of Continuous Flow Activated Sludge Processes to Qualitative Shock Loadings." Journal Water Pollution Control Federation, 38, 85-101 (1966).

122. Krishnan, P., and Gaudy, A. F., Jr., "Studies on the Response of Activated Sludge to Shock Loadings." Biotechnology and Bioengineering, VII, 455-470 (1965).

123. Grady, C. P. L., Jr., and Gaudy, A. F., Jr., Unpublished results.

124. Ribbons, D. W., and Dawes, E. A., "Environmental and Growth Conditions Affecting the Endogenous Metabolism of Bacteria." Annals New York Academy of Sciences, 102, 564-586 (1963).

125. Strange, R. E., Dark, F. A., and Ness, A. G., "The Survival of Stationary Phase Aerobacter aerogenes Stored in Aqueous Suspension." Journal of General Microbiology, 25, 61-76 (1961).

126. Strange, R. E., Wade, H. E., and Ness, A. G., "The Catabolism of Proteins and Nucleic Acids in Starved Aerobacter aerogenes." Biochemical Journal, 86, 197-203 (1963).

127. Dawes, E. A., and Holms, W. H., "Metabolism of Sarcina lutea. III. Endogenous Metabolism." Biochimica et Biophysica Acta, 30, 278-293 (1958).

128. Dawes, E. A., and Ribbons, D. W., "Some Aspects of the Endogenous Metabolism of Bacteria." Bacteriological Reviews, 28, 126-149 (1964).

129. Hoover, S. R., Jasewicz, L., and Porges, N., "Biochemical Oxidation of Dairy Wastes. IV. Endogenous Respiration and Stability of Aerated Dairy Waste Sludge." Sewage and Industrial Wastes, 24, 1144-1149 (1952).

130. Forney, C., and Kountz, R. R., "Activated Sludge Total Oxidation Metabolism." Proceedings 13th Industrial Waste Conference, Purdue University, Lafayette, Indiana, Ext. Ser. 96, 313-320 (1958).

131. Dietrich, S. M. C., and Burris, R. H., "Effect of Exogenous Substrates on the Endogenous Respiration of Bacteria." Journal of Bacteriology, 93, 1467-1470 (1967).

132. Clifton, C. E., "Aging of Escherichia coli." Journal of Bacteriology, 92, 905-912 (1966).

133. Ramsey, H. H., "Endogenous Respiration of Staphylococcus aureus." Journal of Bacteriology, 83, 507-514 (1962).

134. Gaudy, A. F., Jr., "Colorimetric Determination of Protein and Carbohydrate." Industrial Water and Wastes, 1, 17-22 (1962).

135. Standard Methods for the Examination of Water and Waste Water, 12th Edition, American Public Health Association, Inc. (1965).

136. Worthington Manual, Worthington Biochemical Corporation, Freehold, New Jersey (1967).

137. Clark, J. M., Jr., editor, Experimental Biochemistry, W. J. Freeman and Company, San Francisco (1964).

138. Kincannon, D. F., "Study of the Effects of Sodium Chloride on Activated Sludge." Ph.D. Thesis, Oklahoma State University (1966).

139. Neish, A. C., "Analytical Methods for Bacterial Fermentations." Report No. 46-8-3, National Research Council, Canada (1952).

140. Gaudy, A. F., Jr., Komolrit, K., and Gaudy, E. T., "Sequential Substrate Removal in Response to Qualitative Shock Loading of Activated Sludge Systems." Applied Microbiology, 12, 280-286 (1964).

141. Ashwell, G., "Colorimetric Analysis of Sugars." In Methods in Enzymology, Volume III, 73-105, edited by S. P. Colowick and N. O. Kaplan. Academic Press, Inc., New York (1957).

142. Friedeman, T. E., and Haugen, G. E., "Pyruvic Acid. II. The Determination of Keto Acids in Blood and Urine." Journal of Biological Chemistry, 147, 415-422 (1943).

143. Hamilton, I. R., Burris, R. H., and Wilson, P. W., "Pyruvate Metabolism by a Nitrogen-fixing Bacterium." Biochemical Journal, 96, 383-389 (1965).

144. Painter, F. T., and Gaudy, A. F., Jr., Unpublished results.

145. Bergeim, O., "Toxicity of Intestinal Volatile Fatty Acids for Yeast and Escherichia coli." Journal of Infectious Diseases, 66, 222-234 (1940).

146. Wolin, M. J., "Volatile Fatty Acids and the Inhibition of Escherichia coli Growth by Rumen Fluid." Applied Microbiology, 17, 83-87 (1969).

147. Krishnan, P., and Gaudy, A. F., Jr., "Substrate Utilization at High Biological Solids Concentrations." Journal Water Pollution Control Federation, RS 40, R54-66 (1968).

148. Rao, B. S., and Gaudy, A. F., Jr., "Effect of Sludge Concentration on Various Aspects of Biological Activity in Activated Sludge." Journal Water Pollution Control Federation, 38, 794-812 (1966).

149. Moulder, J. W., "Bacterial Metabolism." In Textbook of Microbiology, 105-176. Edited by W. Burrows. W. B. Saunders Co., Philadelphia, Pa. (1968).

150. Wickerham, L. J., "Evidence of the Production of Extracellular Invertase by Certain Strains of Yeasts." Archives of Biochemistry and Biophysics, 76, 439-448 (1958).

151. Dworschack, R. G., and Wickerham, L. J., "Production of Extracellular Invertase by the Yeast, Saccharomyces uvarum NRRL Y-972." Archives of Biochemistry and Biophysics, 76, 449-456 (1958).

152. Rogers, H. J., "The Dissimilation of High Molecular Weight Substances." In The Bacteria, Volume II, 257-318. Edited by I. C. Gunsalus and R. Y. Stanier, Academic Press, New York (1961).

153. Ballard, F. J., "Pathways of Fructose Conversion into Glucose in Foetal Rat Liver." Biochemical Journal, 97, 365-370 (1965).

154. Fraenkel, D. G., "The Phosphoenolpyruvate-initiated Pathway of Fructose Metabolism in Escherichia coli." Journal of Biological Chemistry, 243, 6458-6463 (1968)

155. Loomis, W. F., and Magasanik, B., "Genetic Control of Catabolite Repression of Lac Operon in Escherichia coli." Biochemical and Biophysical Research Communications, 20, 230-234 (1965).

156. Adhya, S., and Echols, H., "Glucose Effect and Galactose Enzymes of Escherichia coli: Correlation Between Glucose Inhibition and Inducer Transport." Journal of Bacteriology, 92, 601-608 (1966).

157. Brower, G., and Gaddis, L., "Filamentous Waste Treatment Systems at Low pH." Journal Water Pollution Control Federation, Research Supplement, 41, R61-72 (1969).

158. Kornberg, H. L., and Elsden, S. R., "Metabolism of 2-Carbon Compounds by Microorganisms." Advances in Enzymology, 23, 401-470 (1961).

159. Stouthamer, A. H., van Boom, J. H., and Bastiaanse, A. J., "Metabolism of C_2 Compounds in Acetobacter aceti." Antonie van Leeuwenhoek Journal of Microbiology and Serology, 29, 393-406 (1963).

160. Stanier, R. Y., Doudoroff, M., and Adelberg, E. A., The Microbial World. Prentice Hall, Inc., Englewood Cliffs, New Jersey (1957).

161. Burkhead, C. E., and McKinney, R. E., "Application of Complete-mixing Activated Sludge Design Equations to Industrial Wastes." Journal Water Pollution Control Federation, 40, 557-570 (1968).

162. Reynolds, P. E., "The Bacterial Cell: Major Structures." In Biochemistry of Bacterial Growth, 73-135. Edited by Joel Mandelstam and K. McQuillen, John Wiley & Sons, Inc., New York (1968).

163. Postgate, J. R., and Hunter, J. R., "Accelerated Death of Aerobacter aerogenes in the Presence of Growth-limiting Substrates." Journal of General Microbiology, 34, 459-473 (1964).

164. Hansen, R. S., and Cox, D. P., "Effect of Different Nutritional Conditions on the Synthesis of Tricarboxylic Acid Cycle Enzymes." Journal of Bacteriology, 93, 1777-1787 (1967).

165. Mandelstam, J., and Halvorson, H., "Turnover of Protein and Nucleic Acid in Soluble and Ribosome Fractions of Non-growing Escherichia coli." Biochimica et Biophysica Acta, 40, 43-49 (1960).

166. Eckenfelder, W. W., Jr., Industrial Water Pollution Control. McGraw Hill Book Company, New York (1966).

167. Pearson, E. A., "Kinetics of Biological Treatment." In Advances in Water Quality Improvement, 381-394. Edited by E. F. Gloyna and W. W. Eckenfelder, Jr., University of Texas Press, Austin (1968).

168. McKinney, R. E., and Symons, J. E., Discussion on "Growth and Endogenous Phases in the Oxidation of Glucose." In Advances in Water Pollution Research, 440-449. Edited by W. W. Eckenfelder. The Macmillan Company, New York (1964).

169. Horwitz, S. B., and Kaplan, N. O., "Hexitol Dehydrogenanses of Bacillus subtilis." Journal of Biological Chemistry, 239, 830-838 (1964).

170. Shaw, D. R. D., "Polyol Dehydrogenases. 3. Galactitol Dehydrogenase and D-Iditol Dehydrogenase." Biochemical Journal, 64, 394-405 (1956).

171. Wolff, J. B., and Kaplan, N. O., "Hexitol Metabolism in Escherichia coli." Journal of Bacteriology, 71, 557-564 (1956).

172. Liss, M., Horwitz, S. B., and Kaplan, N. O., "D-Mannitol-1-Phosphate Dehydrogenase and D-Sorbitol-6-Phosphate Dehydrogenase in Aerobacter aerogenes." Journal of Biological Chemistry, 237, 1342-1350 (1962).

173. Shockley, T. E., and Pride, H. S., "D-Sorbitol-6-Phosphate Dehydrogenase from Lactobacillus casei." Journal of Bacteriology, 77, 695-714 (1959).

174. Stewart, M. J., and Ludwig, H. F., "The Theory of the MAS Waste-water Treatment Process." Part 2. Water and Sewage Works, 109, 97-102 (1962).

175. Stewart, M. J., "Activated Sludge Process Variations, The Complete Spectrum." Water and Sewage Works, 11, 153-158 (1964).

176. Middlebrooks, E. J., and Garland, C. F., "Kinetics of Model and Field Extended Aeration Wastewater Treatment Units." Journal Water Pollution Control Federation, 40, 586-612 (1968).

APPENDIX A

MODEL CALCULATION OF OXYGEN UPTAKE AND RESPIRATION RATES IN CONTINUOUS FLOW EXPERIMENTS

Oxygen consumed by mixed liquor = oxygen in - oxygen out.

$$\text{Oxygen "in," mg/min} = Q_a \times Y_1 \times Z$$

where

Q_a = gas flow rate, liter/min

Y_1 = fraction of O_2 in the influent gas, liter/liter

Z = density of O_2 at 30°C and 760 mm Hg, mg/liter

= 1287 mg/liter

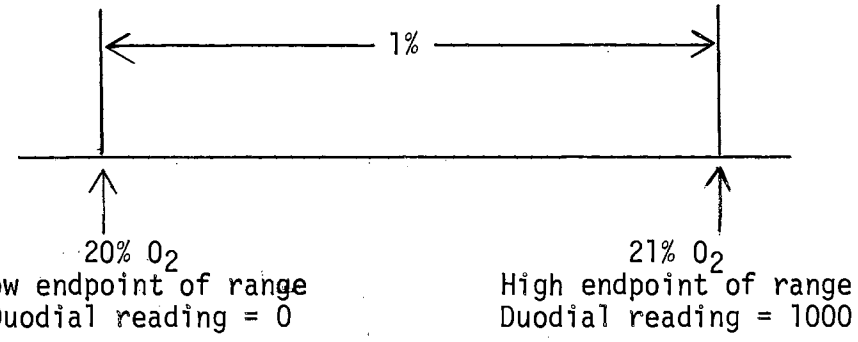
$$\text{Oxygen "out," mg/min} = Q_a \times Y_2 \times Z$$

where

Y_2 = fraction of O_2 in the effluent gas, liter/liter

The fraction of O_2 in the effluent gas (Y_2) was determined using a Beckman Model E2 oxygen analyzer. A sample calculation is shown below:

When air was used as the aerating gas, the range switch of the analyzer was set for 20-21% O_2 range



If the Duodial reading at null balance is 200 for a given sample of effluent gas, the O_2 content of the gas can be computed by using the reading to interpolate between the endpoints of the range used.

$$O_2 \text{ content of gas} = 20\% + \frac{200}{1000}(21\% - 20\%)$$

$$= 20{\cdot}2\%$$

For an airflow rate of 1 liter/min, O_2 uptake rate (mg/min) will be

$$= 1 \times 0.21 \times 1287 - 1 \times 0.202 \times 1287$$

$$= 1287\ (0.21 - 0.202)$$

$$= 1287 \times 0.008$$

$$= 10.3$$

$$O_2 \text{ uptake rate, mg/hr} = 10.3 \times 60 = 618$$

$$O_2 \text{ uptake rate, mg/l/hr} = \frac{O_2 \text{ uptake rate, mg/hr}}{\text{reactor volume, liters}}$$

$$= \frac{618}{3.81} = 162.2$$

Respiration rate, mg/hr/gm dry weight

$$= \frac{O_2 \text{ uptake rate, mg/l/hr}}{\text{sludge dry weight, gm/l}}$$

APPENDIX B

THEORETICAL COD VALUES OF SUBSTRATES AND METABOLIC INTERMEDIATES

Compound	Theoretical COD (mg COD/mg Compound)
Glucose	1.07
Fructose	1.07
Sucrose	1.12
Galactose	1.07
Lactose ($C_{12}H_{22}O_{11} \cdot H_2O$)	1.07
Ribose	1.07
Sorbitol	1.14
Glycerol	1.22
Acetic Acid	1.07
Propionic Acid	1.51
Pyruvic Acid	0.89

VITA

2

G. Jeyasingh Thabaraj

Candidate for the Degree of

Doctor of Philosophy

Thesis: STUDIES ON THE RELEASE OF METABOLIC INTERMEDIATES AND/OR ENDPRODUCTS DURING WASTEWATER PURIFICATION BY ACTIVATED SLUDGE

Major Field: Engineering

Biographical:

Personal Data: Born January 3, 1937, in Tuticorin, Tamil Nadu, India, the son of Gnanamony and Ranjitham.

Education: Attended St. Francis Xavier High School, Tuticorin, 1949-1952; St. John's College, Palayamkottai (India), 1952-1954; graduated from American College, Madurai, University of Madras in 1956, with a Bachelor of Science Degree in chemistry; graduated from A. C. College of Technology, University of Madras in 1959, with a Bachelor of Science Degree in Technology; received the Master of Science Degree from the University of Madras in 1962; completed requirements for the Doctor of Philosophy Degree in Engineering at Oklahoma State University in August, 1969.

Professional Experience: Government of India Research Scholar at the Central Leather Research Institute, Madras (India), from December, 1959, to August, 1961; Senior Research Fellow of the Council of Scientific and Industrial Research at the Central Leather Research Institute, Madras, from September, 1961, to December, 1964; Graduate Research Assistant, School of Civil Engineering, Oklahoma State University, January, 1965, to June, 1969.

Membership in Professional Societies:

Water Pollution Control Federation, American Water Works Association, American Association for the Advancement of Science, Washington Academy of Sciences.

Publications:

Thabaraj, G. J., Bose, S. M., and Nayudamma, Y., "Comparative Studies on the Treatment of Tannery Effluents by Trickling Filter, Activated Sludge, and Oxidation Pond Systems." Bull. Central Leather Research Institute (India), 8, 411-430 (1962).

Thabaraj, G. J., Bose, S. M., and Nayudamma, Y., "Effect of Pre-tanning Operations on the Removal of Globular Proteins and Collagenous Constituents from Hides and Skins." Leather Science, 10, 109-119 (1963).

Thabaraj, G. J., Bose, S. M., and Nayudamma, Y., "Studies on Utilization of Tannery Effluents for Agricultural Purposes." Environmental Health, 6, 1-19 (1964).

Thabaraj, G. J., "Pollution of Natural Waters by Industrial Effluents and Its Control." ALTECH (A. C. College of Technology, Madras, India), 11, 69-79 (1961-1962).

Thabaraj, G. J., and Bose, S. M., "Tannery Effluents and Their Treatment." Lecture Notes on Leather, Asian Productivity Organization, Madras, India (1964).

Thabaraj, G. J., and Gaudy, A. F., Jr., "Effect of Dissolved Oxygen Concentration on the Metabolic Response of Completely Mixed Activated Sludge." Presented at the 24th Purdue Industrial Waste Conference, Purdue University, Lafayette, Indiana, May 6-8, 1969.