RICE UNIVERSITY

STUDIES ON CELL SYNTHESIS IN MIXED MICROBIAL POPULATIONS - LOG PHASE INNOCULA AND TOTAL OXYGEN DEMAND

by

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ABSTRACT

A study of the effects of a log-phase seeding technique and of the oxidative assimilation relations, in soluble substrates, of mixed cultures of bacteria and protozoa, as found in a typical domestic waste water, was conducted in the Environmental Engineering Laboratory of the Rice University during the 1961-1962 academic year. These studies were part of a broad investigation of the study of the Biochemical Oxygen Demand Progression in soluble substrates. This is believed to be the first work using the log-phase seeding technique in shortening the time to the plateau in oxygen utilization and in the formulation of oxidative assimilation relationships in mixed microbial populations.

Previous work on the effects of log-phase seeding and microbial assimilations generally utilized pure cultures and heavy innocula of bacteria with short incubation periods. Substrates studied in this investigation were glucose, glutamic acid, a (1:1) mixture of glucose and glutamic acid, aspartic acid, acetic acid, propionic acid, and lactose. Both Warburg and dilution techniques were utilized.

The log-phase seeding technique produced a marked reduction in lag for all of the substrates and a reduction in time to reach the plateau for lactose, glutamic acid, aspartic acid, and propionic acid. The general pattern of
the progression of biochemical degradation of soluble substrates in shown in graph form. The plateau in oxygen utilization was highly reproducible and was characteristic for a specific substrate. Based on the data obtained, oxidative assimilation equations were developed for the substrates under study. Theoretical plateaus occurred at 41, 40, 39, 44, 50, 52, and 41 per cent of the theoretical oxygen demand in the case of glucose, a (1:1) mixture of glucose and glutamic acid, glutamic acid, aspartic acid, acetic acid, propionic acid, and lactose, respectively.

The nitrogen supplied by the standard dilution technique for B.O.D. analysis is adequate for glucose and lactose concentrations only up to 8 mg/l. At the concentrations of 12.1 mg/l, utilized in this study, the resulting nitrogen deficiency yielded low values for the plateau in oxygen uptake normally representing completion of synthesis.
ACKNOWLEDGEMENTS

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Table of Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>II. Previous Work</td>
<td>6</td>
</tr>
<tr>
<td>III. Theoretical Considerations</td>
<td>13</td>
</tr>
<tr>
<td>IV. Experimental Technique</td>
<td>17</td>
</tr>
<tr>
<td>V. Calculations</td>
<td>20</td>
</tr>
<tr>
<td>VI. Experimental Results</td>
<td>23</td>
</tr>
<tr>
<td>VII. Discussion of Results</td>
<td>41</td>
</tr>
<tr>
<td>VIII. Conclusions</td>
<td>45</td>
</tr>
<tr>
<td>IX. Bibliography</td>
<td>47</td>
</tr>
</tbody>
</table>
**List of Figures**

1. **Relation of Respiration to Assimilation** .......... 2
2. The T.O.D. Concept in the Solids Production - Oxygen Requirement Balance .................. 4
3. **Growth of Parent-Culture and Sub-Cultures of Pneumococcus at 38°C** .......................... 11
4. **Typical B.O.D. Curves-Glucose-Manometric Technique** ........................................ 24
5. **Typical B.O.D. Curves-Glucose and Lactose-Standard Dilution Technique** ................. 25
6. **Typical B.O.D. Curves- Glutamic Acid-Manometric Technique** ................................ 28
7. **Typical B.O.D. Curves- Glutamic Acid-Standard Dilution Technique** ......................... 29
8. **Typical B.O.D. Curves -(1:1) Mixture of Glucose and Glutamic Acid-Standard Dilution Technique** ... 31
9. **Typical B.O.D. Curves-Aspartic Acid-Standard Dilution Technique** .......................... 34
10. **Typical B.O.D. Curves-Propionic Acid-Standard Dilution Technique** ....................... 35
11. **Typical B.O.D. Curves-Lactose-Standard Dilution Technique** .................................. 37
<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I.</strong> Variation in Efficiency of Assimilation of Acetate by Different Species of Organisms</td>
</tr>
<tr>
<td><strong>II.</strong> Variation in Extent of Assimilation of Different Substances by Pure Cultures of Bacteria</td>
</tr>
<tr>
<td><strong>III.</strong> Nitrogen Deficiency of B.O.D. Nutrients in the Standard Methods Technique</td>
</tr>
<tr>
<td><strong>IV.</strong> Plateau B.O.D. Values and Theoretical Cell-Yield</td>
</tr>
<tr>
<td><strong>V.</strong> Effects of Log Phase Seeding</td>
</tr>
</tbody>
</table>
INTRODUCTION

One of the most effective methods of removing soluble organics from liquid waste streams involves the use of aerobic biological oxidation. A sound knowledge of the basic relationships involved in the aerobic biochemical degradation mechanisms will greatly aid not only in the proper delineation of the progression of the biochemical oxygen demand (B.O.D.), but also in development of a rational approach toward design and operational criteria for the waste treatment processes.

A fundamental approach to the design of an aerobic biological oxidation process requires an understanding of the partitioning of the soluble organic substrate by the inter-related processes of respiration and synthesis, as shown in Figure 1. Synthesis represents the conversion of a part of the substrate into new biological protoplasm and the energy needed is usually furnished to the system by oxidation of a portion of the substrate. Hence, a part of the organic matter removed from the liquid waste is converted into new cell material of the empirical chemical formulation C_5H_7NO_2 as reported by Porges\(^{(1)}\) while another fraction is oxidized to carbon dioxide and water. A definition of such fractionation of the substrate and the related kinetics help in pre-
FIG. 1 - RELATION of RESPIRATION to ASSIMILATION

SOLUBLE ORGANIC COMPOUNDS + [BACTERIA OXYGEN NUTRIENTS] → RESPIRATION (OXIDATION)

[CARBON DIOXIDE WATER ENERGY]

ASSIMILATION (SYNTHESIS)

NEW BACTERIAL CELL MATERIAL
dicting, for a treatment process, the oxygen requirements and the quantity of cells produced.

In 1958, Busch\(^{(2)}\) established a relation between the plateau values of the oxygen uptake curve and the theoretical B.O.D. (T.O.D.) for several pure compounds using mixed microbial populations. Recent investigations\(^{(3,4)}\) from the laboratory of Environmental Engineering of the Rice University have delineated the oxidation-assimilation relationships for glucose and have demonstrated the solids production reactions over the broad range of biological environments. The T.O.D. test of Busch\(^{(4)}\) involves the measurement of oxygen uptake to the plateau representing completion of synthesis and the resulting cell-growth thereat (Figure 2).

Working with the substrates, glucose, glutamic acid, and a mixture of 1:1 glucose and glutamic acid, Busch found that the plateau in oxygen utilization representing the conversion of all the substrate to cell material occurs in 12 to 48 hours (depending on the analytical environment) and has a characteristic value for a given substrate. Shortening of the time required to attain the plateau in oxygen utilization would be desirable.

The present investigation was undertaken to study attaining the plateau in less time by employing a log-phase seeding technique and to formulate the theoretical oxidation
FIG. 2 - THE T.O.D. CONCEPT IN THE SOLIDS PRODUCTION-OXYGEN REQUIREMENT BALANCE

T.O.D., mg/l. = \frac{T.O.D. - \text{PLATEAU B.O.D.}}{1.414} \text{ gm/gm}

MAXIMUM SOLIDS PRODUCTION

PLATEAU B.O.D. VALUE

MINIMUM OXYGEN REQUIREMENTS FOR COMPLETE BIO-OXIDATION OF SOLUBLE ORGANICS

T.O.D. - \text{UNEXERTED}

B.O.D. - \text{EXERTED}

TIME
equations for a number of chemicals representative of the major classes of soluble organic compounds.
PREVIOUS WORK

The investigations of Barker\(^{(5)}\) constitute a landmark in the studies of microbial assimilations. Using non-proliferating cells of the colorless alga, *Prototheca zopfii* in manometric experiments of 1 to 4 hours duration, Barker observed quantitative relationships between the quantity of substrate dissimilated and the quantity assimilated. With acetic acid as the substrate the oxygen consumption was 50% of the theoretical value for complete combustion; the corresponding figures for other substrates being 58 for propionic acid and 30 for glucose, without blank-correction. Barker concluded "... the process of assimilation of these compounds proceeds in two experimentally distinct stages. The first, which has been called the primary process of assimilation, consists of an oxidative conversion of the substrate into a carbohydrate which is stored in the cell as glycogen. The formation of this carbohydrate is rapid compared to its subsequent decomposition in the process of cell-synthesis which constitutes the second state of assimilation." Later investigations of Giesberger\(^{(6)}\), Clifton\(^{(7)}\), Doudoroff\(^{(8)}\), Busch\(^{(2)}\), and other workers in the field have further confirmed the conclusions of Barker.

While working with the manometric technique on the oxidative assimilation of sugars and related substances by *Pseudomonas saccharophila*, Doudoroff\(^{(8)}\) observed that
the disappearance of the original substrate can usually be detected by more or less sharp decrease in the rate of oxygen uptake. The actual amount of oxygen used, and of CO₂ produced in this initial conversion of the substrate can thus be estimated. In most cases, the 'break' in the rate of oxygen consumption could be clearly shown, and unambiguously interpreted as a shift from the breakdown of the substrate to respiration of the cell-materials (autotrespiration)."

Working with Pseudomonas calco-acetica and Escherichia coli, in the presence of different substrates, Clifton found that regardless of the initial concentration of the substrate, or of organisms, and of the presence of end products of respiration in the suspension medium, the same general pattern of oxidative assimilation was observed.

Recent investigations by Busch and Myrick have delineated the relationship between synthesis and respiration of glucose in mixed microbial populations. The variation in efficiency of the oxidative assimilation of acetate by different species of organisms, as shown in Table I is interesting. Similar variation in efficiency of oxidation of different substrates by each of the two species of microorganisms as presented in Table II, is shown by the per cent of the substrate oxidized in each case. One of
the aspects of this investigation is to establish such relationships using different classes of organic compounds and mixed microbial populations.

Working with *Pneumococcus* in 1916, Chesney\(^{(13)}\) demonstrated the effect of age of the parent culture on the lag phase. He showed that only the subculture made from the exponential phase had no observable delay in the rate of multiplication in the fresh medium; and the rate of growth of this subculture paralleled that of the parent culture (Figure 3). Investigations by Hinshelwood\(^{(14)}\) and others in the field have confirmed these results.

Hinshelwood\(^{(14)}\) ascribed the reduction in lag in a fresh media to the carry-over with subculture inoculum of a diffusible substance produced during the early lag phase of the parent culture. Chesney\(^{(13)}\) theorized that some of the cells of *Pneumococcus* were poisoned by their own metabolic products, the toxic substance gradually reaching a peak at the end of the growth phase. Thus lag was considered as "an expression of injury which the bacterial cell has sustained from its previous environment."

Although Gaffney and Heukelekian\(^{(15)}\) used what may be termed a log-phase seeding technique, their work involved extremely high substrate concentrations and is thus not directly comparable to the current study.
TABLE I

Variation in Efficiency of Assimilation of Acetate by Different Species of Organisms

1) Chlorella pyrenoidesa, Prototheca zopfii, Candida albicans and different spirilla
   \[ \text{CH}_3\text{COOH} + \text{O}_2 \rightarrow (\text{CH}_2\text{O}) + \text{CO}_2 + \text{H}_2\text{O} \]

2) Saccharomyces cerevisiae
   \[ 3 \text{CH}_3\text{COOH} + 4 \text{O}_2 \rightarrow 2 (\text{CH}_2\text{O}) + 4 \text{CO}_2 + 4 \text{H}_2\text{O} \]

3) Escherichia coli and Pseudomonas calco-acetica
   \[ 2 \text{CH}_3\text{COOH} + 3 \text{O}_2 \rightarrow (\text{CH}_2\text{O}) + 3 \text{CO}_2 + 3 \text{H}_2\text{O} \]
## TABLE II

Variation in Extent of Assimilation of Different Substrates by Pure Cultures of Bacteria

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th><strong>Escherichia coli</strong>&lt;sup&gt;(11)&lt;/sup&gt;</th>
<th>% Substrate Oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>$\frac{1}{3} 3 \text{O}_2 \rightarrow 3(\text{CH}_2\text{O})$</td>
<td>50</td>
</tr>
<tr>
<td>Succinate</td>
<td>$\frac{1}{2.5} 2 \text{O}_2 \rightarrow (\text{CH}_2\text{O})$</td>
<td>72</td>
</tr>
<tr>
<td>Fumarate</td>
<td>$\frac{1}{2} 2 \text{O}_2 \rightarrow (\text{CH}_2\text{O})$</td>
<td>80</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>$\frac{1}{1.5} 1.5 \text{O}_2 \rightarrow (\text{CH}_2\text{O})$</td>
<td>50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th><strong>Bacillus Subtilis</strong>&lt;sup&gt;(12)&lt;/sup&gt;</th>
<th>% Substrate Oxidized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>$\frac{1}{3} 3 \text{O}_2 \rightarrow 3(\text{CH}_2\text{O})$</td>
<td>50</td>
</tr>
<tr>
<td>Succinate</td>
<td>$\frac{1}{4} 4 \text{O}_2 \rightarrow 3(\text{CH}_2\text{O})$</td>
<td>57</td>
</tr>
<tr>
<td>Fumarate</td>
<td>$\frac{1}{4} 4 \text{O}_2 \rightarrow 5(\text{CH}_2\text{O})$</td>
<td>44</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>$\frac{1}{0.5} 0.5 \text{O}_2 \rightarrow 2(\text{CH}_2\text{O})$</td>
<td>40</td>
</tr>
</tbody>
</table>
FIG. 3 - GROWTH of PARENT CULTURE (P) and SUBCULTURES (S₁ - S₈) of PNEUMOCOCCUS at 38°C, (13)
One or more of the several factors such as the threshold concentrations of the source of energy, a specific organic or inorganic nutrient, toxic metabolite or oxygen per cell may be primarily responsible for control of growth-mechanisms. Clifton\textsuperscript{(16)} pointed out "the need of the cell for essential metabolites, and also for the complex relationships that must exist between diffusion of substances into and out of the cell, the synthetic reactions, and the formation of cellular elements." Thus a log-phase seeding technique appears to be useful in acceleration of oxygen uptake by significantly decreasing the lag period.
THEORETICAL CONSIDERATIONS

In a series of investigations on the biochemical degradation of soluble organics, Busch\(^2\) has presented the progression of B.O.D. exertion for several pure substances and the oxidation-assimilation relationships for glucose and has demonstrated the validity of solids production reactions over the broad range of biological environments. He has evolved a total oxygen demand test which involves the measurement of the B.O.D. value at the plateau in oxygen utilization and the resulting cell weight thereat.

Attainment of the plateau in oxygen utilization implies conversion of all the substrate into new protoplasm of the empirical chemical formulation \(C_5H_7NO_2\) with a theoretical oxygen demand of 1.414 gm/gm. Thus, at the plateau, the difference between the T.O.D. of the original substrate and the exerted B.O.D. is contained in the cell material. Conversely, for any given waste, measurement of the B.O.D. at the plateau followed by gravimetric or chemical determination of the resulting cell-growth should yield the total oxygen demand value of the substrate. Thus oxidative-assimilation relationships are of fundamental significance from the standpoint of waste characterization and materials balances.

In establishing a correlation between the plateau value in oxygen utilization in the B.O.D. bottle and the
theoretical B.O.D. for a given substance, Busch found that, with soluble organic substrates, the plateau, representing the conversion of all the substrate into new cell material, occurs in 12 to 48 hours, depending on the analytical environment. Since oxygen uptake of microbial populations follows approximately the pattern of growth curves, the plateau represents the end of the growth phase. The time required for attainment of this plateau is dependent in large part upon the duration of the lag period during which the metabolism of the organism is gradually building up to a steady state and thus becomes adapted for growth and multiplication at a maximal rate under the particular conditions of the environment. Thus it is apparent that evolving a means of decreasing this lag would result in attainment of the plateau in less time and hence an improved technique in the application of the T.O.D. test.

A study of the microbiological literature reveals that the variations in lag period are mostly dependent upon the physiological age of the culture from which the inoculum is taken. Working with Pneumococcus in 1916, Chesney observed that the inoculum from the latent lag showed no immediate growth on transfer to another medium of the same composition as used for the cultivation of the parent organism. Seed transferred from the later part of the lag
showed some growth in 2 hours, while the subculture taken from the exponential phase showed no observable delay in growth in the fresh medium and the rate of growth was parallel to that of the parent culture (Figure 3). The transplantations from the later portions of the stationary phase showed some lag and only a small amount of growth. These results indicate that a significant reduction in lag can be obtained only by using a log-phase seeding technique.

The phenomena of reduction in lag by this technique is ascribed to the mechanisms of adaptation of the mixed microbial populations. This adaptation probably consists in synthesis and accumulation of enzymatic equipment required for the most efficient utilization of the substrate medium. Adaptation of the log-phase seeding technique may possibly confer certain benefits on the T.O.D. test. These are a decrease in the lag and a consequent reduction in time to attain the plateau in oxygen utilization; and production of a consistent seed, by using for the inoculum the species that are best adapted to the substrate, produced by natural selection from the original biota of mixed microbial organisms.

The investigations reviewed so far relate to the study of growth mechanisms in pure cultures. Little information is available on the effect of competition of the various species
in a mixed culture on overall growth patterns. The significance of studying mixed culture growth characteristics is that pure cultures do not exist under natural conditions and cannot be maintained in a treatment process. A definitive disclosure of the stoichiometric biochemical reactions involved in the degradation of pure soluble organics by mixed microbial populations would also contribute to the basic knowledge of the field.
EXPERIMENTAL TECHNIQUE

The B.O.D. measurements made in this investigation employed the standard dilution and Warburg techniques at 20°C. Concentrations of substrates usable in the Warburg technique correspond to the strength of an actual waste. The bottle dilution technique requires low concentrations of the substrate corresponding to those present in natural stream conditions. Thus the techniques employed cover a wide range of biological environments, from the waste treatment process to the polluted water courses. Determination of cell weight at the plateau using membrane filters was carried out as outlined in a recent publication\(^4\). Determinations of pH were made at the beginning and at the termination of the experiments. Unless otherwise stated, the pH was maintained at a value of 7.0 ± 0.4 throughout the runs. Analyses for oxygen uptake were often made at very short intervals throughout the day and night as required for uptake definition.

A. PROCEDURE FOR DETERMINING THE B.O.D. VALUES BY MANOMETRIC TECHNIQUE

The manometric technique employed in this investigation was based on the procedure outlined in Standard Methods\(^17\).
For seeding purposes, sewage collected from the West University Place and Bellaire treatment plants was settled at 20°C for 24 hours in the incubation chamber. After aging the seed for 1 day, the supernatant was filtered through Whatman No. 2 paper as suggested by Busch. As recommended by Sawyer, mineral nutrient ratios of B.O.D. to nitrogen of 17:1 and B.O.D. to phosphorous ratio of 90:1 were maintained in all the experiments. Two hundred ml/1 of the filtered sewage seed was used for the initial curves throughout the runs. In order to maintain an approximately uniform initial population in the log-seeding curves, 80, 40 and 28 mg/l of seed was transferred from points on the initial curve representing 10, 20 and 30% of T.O.D. exertion. Where the points of transfer were different, proportional volumes of seed were used for the subcultures.

B. PROCEDURE FOR DETERMINING B.O.D. VALUES - BOTTLE DILUTION TECHNIQUE

The procedure employed in the bottle dilution technique was based on the methods outlined in the Standard Methods. Sewage was obtained from two different sources and the seed preparation was as described under the manometric technique. To the dilution water was fed 1 ml of each of the 4 standard B.O.D. nutrient solutions and 5 ml of filtered sewage seed per liter of dilution water.
For log-phase inocula, 5 ml. of the diluted sample containing the adapted population were used per litre of dilution water. For the oxygen analysis of the samples, the Alsterberg alkaline-iodide sodium azide modification of the Winkler method\(^{(17)}\) was used.
A. COMPUTATION OF B.O.D. VALUES - MANOMETRIC TECHNIQUE

An equation (1) for calculating biochemical oxygen demand from the manometer deflections was derived by Langlier and Caldwell (19).

1) \( W = Kh \)

where \( W \) is oxygen uptake in mg/L

\( h \) is the observed manometer deflections after correction for thermobarometric changes (in cm.)

\( K \) is the temperature-fluid constant

The value of the temperature-fluid constant (k) may be taken as

2) \( K = \left( \frac{1430}{P_o V_s} \right) \left( 273 V_g / T + V_f \right) \cdot C \)

where \( V_g \) is the total volume of gas in flask, connection, and manometer to the index-point (in ml.)

\( V \) is the volume of original sample being tested (in ml.)

\( V_f \) is the total volume of fluid (sample plus added dilutents, nutrients, seed, etc.) undergoing testing in the flask (in ml.)

\( C \) is the solubility of oxygen under test conditions (in ml/ml) = 0.028 ml/ml at 25°C or 0.031 ml/ml at 20°C

\( P_o \) is the normal pressure of manometer fluid (in cm.)
= (height of manometer fluid equal to atmospheric pressure)
= 76 \times 13.6 / \rho

where \( \rho \) is the specific gravity of manometer fluid; for Brodies solution \( \rho = 1.030 \)

T is temperature (in degrees absolute)

Employing Brodies solution for the manometer fluid and the temperature of the constant temperature bath at 20°C, the value of "K" may be taken as

\[ K = 1.314 \frac{V_g}{V_s} + 0.04402 \frac{V_f}{V_s} \]

Therefore

3) \[ W = h[1.314 \frac{V_g}{V_s} + 0.04402 \frac{V_f}{V_s}] \]

Where the ratio of \( \frac{V_f}{V_s} \) or the "secondary dilution factor" is small, equation 3) may be reduced to

4) \[ W = 1.31h[\frac{V_g}{V_s}] \]

The symbols used in these equations are those of Ludwig, Oswald, and Gotaas (20).

B. computation of b.o.d. values - BOTTLE DILUTION TECHNIQUE

An equation (5) for calculating the biochemical oxygen demand of samples analysed using the dilution technique in bottles was derived by Busch and Sawyer (21).

5) \[ \text{mg/L B.O.D.} = [D.O_{s}-(D.O_{b} - D.O_{ib}) - D.O_{is}](1/P) \]
where $D.O_s = \text{mg/L dissolved oxygen in zero day diluted sample}$

$D.O_b = \text{mg/L dissolved oxygen in zero day diluted blank}$

$D.O_{ib} = \text{mg/L dissolved oxygen in incubated blank}$

$D.O_{is} = \text{mg/L dissolved oxygen in incubated sample}$

$P = \text{per cent of substrate expressed as a decimal}$

The equation in this form is applicable only to substrates representing a small fraction of the total volume of the individual B.O.D. bottles.
EXPERIMENTAL RESULTS

A. GLUCOSE

The B.O.D. values obtained with the manometric technique for mixed microbial populations, in the presence of glucose, are plotted as a function of time in Figure 4. As shown by the oxygen uptake curve, after an initial lag, the biochemical oxygen demand proceeds at a relatively rapid rate, and on reaching a value characteristic for the substrate, decreases to a rate near that of the control sample. This change in the rate of respiration or plateau value corresponds to the depletion of the substrate. Similar curves obtained with the bottle dilution technique are presented in Figure 5. It was observed from Figures 4 and 5 that the lag period was appreciably reduced by log-phase seeding while the time to attain the plateau remained undiminished. The points of seed transfer from the exponential phase of the initial curve are indicated by the arrows marked in Figures 4 and 5. The oxidative-assimilation relationships defined by Busch (9) are presented below:

\[ 24 \text{C}_6\text{H}_{12}\text{O}_6 + 54\text{O}_2 + 12\text{NH}_3 \rightarrow 30\text{CH}_2\text{O} + 12\text{C}_5\text{H}_7\text{NO}_2 + 54\text{CO}_2 + 90\text{H}_2\text{O} \]

\[ 30 \text{CH}_2\text{O} + 50\text{O}_2 + 5\text{NH}_3 \rightarrow 5\text{C}_5\text{H}_7\text{NO}_2 + 5\text{CO}_2 + 20\text{H}_2\text{O} \]

\[ 24 \text{C}_6\text{H}_{12}\text{O}_6 + 59\text{O}_2 + 17\text{NH}_3 \rightarrow 17\text{C}_5\text{H}_7\text{NO}_2 + 59\text{CO}_2 + 110\text{H}_2\text{O} \]
Substrate: Glucose
Warburg Technique (W-18)
W.U.P. Seed Filtered thru Whatman no. 2 paper

(1)(2)(3) Points of Log-Seed Transfer and Resulting Curves

FIG. 4 - TYPICAL B.O.D. CURVES at 20°C - LOG. PHASE SEEDING
Substrates, T.O.D.: 12.9 mg./l.
- Glucose
- Lactose
Plateau B.O.D.: 5.10 mg./l.
Common Seed

FIG. 5 - TYPICAL B.O.D. CURVES at 20°C - LOG. PHASE SEEDING

Dilution Technique (Rx-50, 51)
From this equation it is observed that the respiratory quotient has a value of 1.0. Based on a substrate concentration of 8.06 mg/L, the theoretical plateau B.O.D. value equals 3.54 mg/L or 41.1% of the T.O.D. and the total cell-yield is 0.416 grams per gram of theoretical oxygen demand.

The results obtained on 10 experiments in this study are presented below:

Concentration of Glucose substrate: 12.1 mg/L
T.O.D. of Glucose substrate: 12.9 mg/L
Observed Average Plateau B.O.D.: 5.07 mg/L

For the substrate concentration utilized in this study, the plateau B.O.D. should equal 5.30 mg/L or 41.1% of the T.O.D. Since the observed average plateau was only 5.07 mg/L, a deficiency of nitrogen was suspected and the calculations made to check this question are presented in Table III. The combined data of the equations of Busch(9) which were experimentally substantiated by a materials balance(4) and the demonstrated nitrogen deficiency satisfactorily explain the low plateau obtained in this study.

B. GLUTAMIC ACID

Typical B.O.D. curves with glutamic acid as the substrate are presented in Figure 6, for the Warburg technique and in Figure 7 for the dilution technique. With the Warburg technique, there was a considerable reduction in lag,
while the time to reach the plateau remained undiminished. With the dilution technique there was considerable reduction in lag and also reduction in time to reach the plateau. From the data obtained on 10 experiments the oxidative-assimilation equations may be deduced thus:

Concentration of glutamic acid: 12.1 mg/L
T.O.D. of glutamic acid: 11.8 mg/L

Observed Average Plateau B.O.D. = 4.65 mg/L

Equations for cell-synthesis:

\[ 24 \text{C}_5\text{H}_9\text{NO}_4 + 42 \text{O}_2 \rightarrow 6 \text{CH}_2\text{O} + 12 \text{C}_5\text{H}_7\text{NO}_2 + 54 \text{CO}_2 + 42 \text{H}_2\text{O} + 12 \text{NH}_3 \]

\[ 6 \text{CH}_2\text{O} + \text{O}_2 + \text{NH}_3 \rightarrow \text{C}_5\text{H}_7\text{NO}_2 + \text{CO}_2 + 4 \text{H}_2\text{O} \]

\[ 24 \text{C}_5\text{H}_9\text{NO}_4 + 43 \text{O}_2 \rightarrow 13 \text{C}_5\text{H}_7\text{NO}_2 + 55 \text{CO}_2 + 46 \text{H}_2\text{O} + 11 \text{NH}_3 \]

The equation indicates a respiratory quotient value of 1.3. The theoretical plateau B.O.D. value is 4.62 mg/L or 39% of the T.O.D. A total yield of 0.424 grams of cells per gram of theoretical oxygen demand is observed.

TABLE III

Nitrogen Deficiency of B.O.D. Nutrients of the Standard Methods

Technique

1) Using 1 ml of each of the B.O.D. nutrient solutions per liter of dilution water, Nitrogen content provided = \((14/53.5)(1.7) = 0.445 \text{ mg/l} \)

2) Using a substrate concentration of glucose or lactose at 8.06 mg/l
Substrate: Glutamic Acid
Warburg Technique (W-19)
W.U.P. Seed Filtered thru Whatman no. 2 Filter Paper
①,②,③ Points of Log-Seed Transfer and Resulting Curves

FIG. 6—TYPICAL B.O.D. CURVES at 20°C—LOG. PHASE SEEDING
FIG. 7 — TYPICAL B.O.D. CURVES at 20°C—LOG.
PHASE SEEDING
Cell yield = 8.06 x 1.07 x 0.416 = 3.56 mg/l

Nitrogen required = \((14/113) \times 3.56\) = 0.445 mg/l

3) Using a substrate concentration of glucose or lactose at 12.09 mg/l,

Cell yield = 12.09 x 1.07 x 0.416 » 5.35 mg/l

Nitrogen required = \((14/113) \times 5.35\) = 0.67 mg/l

Thus, at concentrations of glucose or lactose greater than 8.06 mg/l, a deficiency in nitrogen requirements is evident using standard dilution technique as outlined in Standard Methods. (17)

C. (1:1) MIXTURE OF GLUCOSE AND GLUTAMIC ACID

Typical curves obtained with the dilution technique are presented in Figure 8. A considerable reduction in lag was noted. The growth rate declined gradually past the inflection point with the result that the time to attain the plateau remained undiminished. The earlier portion of the growth curve was typical of glutamic acid while the later portion was typical of glucose. From the data obtained from 10 experiments, the oxidative assimilation equations may be derived thus:

Concentration of the substrate: 12.1 mg/L; T.O.D. = mg/L
FIG. 8 - TYPICAL B.O.D. CURVES at 20°C - LOG. PHASE SEEDING

- Dilution Technique (S-7)
- Substrate: (1:1) Glucose-Glut. Acid T.O.D.: 12.5 mg./l.
- Plateau B.O.D.: 4.96 mg./l.
Observed Average Plateau B.O.D. = 4.86 mg/L

Equations for cell-synthesis:

\[
294 \text{C}_6\text{H}_{12}\text{O}_6 + 360 \text{C}_5\text{H}_9\text{NO}_4 + 1302 \text{O}_2 \rightarrow 282 \text{CH}_2\text{O} + 360 \text{C}_5\text{H}_7\text{NO}_2 + 1482 \text{CO}_2 + 1842 \text{H}_2\text{O}
\]

\[
282 \text{CH}_2\text{O} + 47 \text{O}_2 + 47 \text{NH}_3 \rightarrow 47 \text{C}_5\text{H}_7\text{NO}_2 + 47 \text{CO}_2 + 188 \text{H}_2\text{O}
\]

\[
294 \text{C}_6\text{H}_{12}\text{O}_6 + 360 \text{C}_5\text{H}_9\text{NO}_4 + 1349 \text{O}_2 + 47 \text{NH}_3 \rightarrow 407 \text{C}_5\text{H}_7\text{NO}_2 + 1529 \text{CO}_2 + 2030 \text{H}_2\text{O}
\]

The equation indicates a respiratory quotient value of 1.17.
The theoretical plateau B.O.D. value is 4.95 mg/L or 39.6% of the T.O.D. A total yield of 0.42 grams of cells per gram of theoretical oxygen demand is noted.

D. ASPARTIC ACID

Typical B.O.D. curves obtained with the dilution technique are presented in Figure 9. It was observed that there was a reduction both in lag and the time to attain the plateau, as noted for glutamic acid. From the data obtained on 10 experiments, the cell-synthesis equations may be deduced thus.

Concentration of Aspartic Acid: 16.1 mg/L; T.O.D. = 11.6 mg/L

Observed Average Plateau B.O.D. = 4.90 mg/L

Equations for cell-synthesis:
The equation indicates a respiratory quotient of 1.75. The theoretical plateau B.O.D. value is 5.14 mg/L or 44.3% of the T.O.D. A total yield of 0.392 grams of cells per gram of theoretical oxygen demand is indicated.

E. PROPIONIC ACID

Typical B.O.D. curves obtained with the dilution technique are presented in Figure 10. It was observed that there was a considerable reduction in lag and also a reduction in time to reach the plateau. From the data obtained in 8 experiments using the dilution technique, oxidative-assimilation equations may be deduced as follows.

Concentration of Propionic Acid: 6.05 mg/L  T.O.D. = 9.19 mg/L

Observed Average Plateau B.O.D. = 4.32 mg/L

Equations for cell-synthesis:

12 C₄H₇NO₄ + 15 O₂ → 6 CH₂O + 3 C₅H₇NO₂ + 27 CO₂ + 12 H₂O + 9 NH₃

12 C₄H₇NO₄ + 16 O₂ → 4 C₅H₇NO₂ + 28 CO₂ + 16 H₂O + 8 NH₃
FIG. 9—TYPICAL B.O.D. CURVES at 20°C-LOG.
PHASE SEEDING

Dilution Technique (Rx-28)

Substrate: Aspartic Acid
T.O.D.: 11.6 mg./l.
Plateau B.O.D.: 4.85 mg./l.

TRANSFER POINT
FIG. 10 — TYPICAL B.O.D. CURVES at 20°C-CLOG.

PHASE SEEDING

Substrate: Propionic Acid
T.O.D.: 9.19 mg/l.
Plateau: 4.80 mg/l.

Dilution Technique (Rx-48)

TIME—HOURS

0  8  16  24  32

TIME—HOURS

0  8  16  24  32

B.O.D.—mg/l.
The equation indicates a respiratory quotient of 0.73. The theoretical plateau B.O.D. value is 4.84 mg/L or 52.6% T.O.D. A total yield of 0.336 grams of cells per gram of theoretical oxygen demand is indicated.

F. ACETIC ACID

Although variable lag periods and variable effectiveness of log-seeding were noted, the plateau in oxygen utilization was highly reproducible. From the data obtained on 10 experiments using the dilution technique, oxidative-assimilation equations may be deduced as follows.

Concentration of acetic acid: 6.05 mg/L; T.O.D. = 6.45 mg/L

Observed Average Plateau B.O.D. = 3.24 mg/L

Equations for cell-synthesis:

\[
\begin{align*}
96 \text{C}_2\text{H}_4\text{O}_2 & \rightarrow 96 \text{O}_2 \rightarrow 18 \text{NH}_3 \rightarrow 6 \text{CH}_2\text{O} \rightarrow 18 \text{C}_5\text{H}_7\text{NO}_2 \rightarrow 96 \text{CO}_2 \rightarrow 150 \text{H}_2\text{O} \\
6 \text{CH}_2\text{O} & \rightarrow \text{O}_2 \rightarrow \text{NH}_3 \rightarrow \text{C}_5\text{H}_7\text{NO}_2 \rightarrow \text{CO}_2 \rightarrow 4 \text{H}_2\text{O}
\end{align*}
\]

\[
96 \text{C}_2\text{H}_4\text{O}_2 \rightarrow 97 \text{O}_2 \rightarrow 19 \text{NH}_3 \rightarrow 19 \text{C}_5\text{H}_7\text{NO}_2 \rightarrow 97 \text{CO}_2 \rightarrow 154 \text{H}_2\text{O}
\]

The equation indicates a respiratory quotient of 1.0. The theoretical plateau B.O.D. value is 3.26 mg/L or 50.5% of the T.O.D. A total yield of 0.35 grams of cells per gram of theoretical oxygen demand is indicated.

G. LACTOSE

Typical B.O.D. curves for the dilution technique are presented in Figure 11. It was observed that there was a reduction in lag and also a reduction in time to attain the
Substrate: Lactose
T.O.D.: 12.9 mg/l.
Plateau B.O.D.: 5.10 mg/l.

FIG. 11—TYPICAL B.O.D. CURVES at 20°C—LOG.
PHASE SEEDING
plateau. The cell-synthesis equations are the same as for glucose.

H. LACTOSE VS. GLUCOSE

A comparison of the progression of biological degradation of a monosaccharide and a disaccharide was made, with the dilution technique, using glucose and lactose and a common seed. The B.O.D. curves obtained are presented in Figure 4.

With glucose, although a reduction in lag was obtained, the time to reach the plateau was not diminished with the log-phase seeding. Since lactose is a disaccharide, it may involve hydrolysis, with the formation of glucose and galactose. A reduction in lag and also a reduction in time to reach the plateau was obtained for lactose with log-phase seeding.

A summary of the experimental results for plateau B.O.D. values and lag periods of the various substrates are presented in Tables IV and V.
### TABLE IV

Plateau B.O.D. Values and Theoretical Cell-Yield
(with normal and log-phase seeding)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>No. of Expts.</th>
<th>Conc. used (mg/l)</th>
<th>T.O.D. (mg/l)</th>
<th>Plateau B.O.D. Values (mg/l)</th>
<th>Cell-yield in gm/gm of T.O.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Theoretical</td>
<td>Avg. Recovered</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td>12.1</td>
<td>12.9</td>
<td>5.30</td>
<td>5.10*</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>10</td>
<td>12.1</td>
<td>11.9</td>
<td>4.62</td>
<td>4.65</td>
</tr>
<tr>
<td>Mixture</td>
<td>10</td>
<td>12.1</td>
<td>12.5</td>
<td>4.95</td>
<td>4.86</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>10</td>
<td>16.1</td>
<td>11.6</td>
<td>5.14</td>
<td>4.90</td>
</tr>
<tr>
<td>Lactose</td>
<td>6</td>
<td>12.1</td>
<td>12.9</td>
<td>5.30</td>
<td>5.07*</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>8</td>
<td>6.05</td>
<td>9.19</td>
<td>4.84</td>
<td>4.82</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>10</td>
<td>6.05</td>
<td>6.45</td>
<td>3.26</td>
<td>3.24</td>
</tr>
</tbody>
</table>

* Nitrogen Limiting; see Table III
TABLE V
Effects of Log-Phase Seeding
(From Figures 5, 7, 8, 9, 10 & 11)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reduction (in hours) in:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lag</td>
<td>Time to Plateau</td>
</tr>
<tr>
<td>Glucose</td>
<td>7</td>
<td>insignificant</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>1:1 Mixture, Glucose and Glutamic Acid</td>
<td>7</td>
<td>insignificant</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Lactose</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>
DISCUSSION OF RESULTS

The results obtained from the experiments presented clearly demonstrate the validity of formulating stoichiometric equations for the oxidative assimilation of soluble substrates in mixed microbial populations based on the oxygen uptake measured experimentally. The data presented in Tables IV and V indicate that acceleration of the total oxygen demand was effected in the case of all of the soluble substrates except glucose and the mixture of (1:1) glucose and glutamic acid. A reduction in lag was noted in all the log-seed curves developed upon transplantation of the seed from a point in the exponential phase of the initial curve. Further, the data indicate that the highly reproducible plateau in oxygen utilization is characteristic for a specific substrate. It was further observed that the bottle dilution technique gave more reproducible results than the Warburg technique. It is gratifying to note consistent results obtained by using sewage seed from different sources.

The data presented in Table IV indicate actual plateau B.O.D. values of 5.10 and 5.07 mg/L for glucose and lactose as compared to the theoretical values of 5.30 mg/L. Previous workers (9) demonstrated that a very highly reproducible plateau occurs at 41.1% of the T.O.D. (5.3 mg/L). A deficiency of nitrogen was suspected and the calculations made to check
this question are presented in Table III. It was found that at substrate concentrations higher than 8.06 mg/L of glucose or lactose, nitrogen was deficient.

It is interesting to note that in spite of a considerable reduction in lag, the time to attain the plateau in glucose oxidation remained undiminished by using the log-phase seeding technique. This phenomena may be due to the inherent characteristics of the substrate itself.

With glutamic acid as the substrate, the lag was considerably reduced as well as the time to attain the plateau. The data obtained and the theoretical stoichiometric equations developed for synthesis are in agreement. Similar results on dissimilation of glutamic acid were reported by Gerhardt(22) et al., using strains of Brucella abortus.

With a mixture of (1:1) glucose and glutamic acid, a reduction in lag was observed, but the time to attain the plateau remained undiminished. The growth pattern noted was representative of the individual growth patterns of the substrate components. The data obtained and the theoretical synthesis equations developed are in full agreement. A recent publication(4) from the laboratory of Environmental Engineering at Rice University contains materials balance data obtained to substantiate the validity of the synthesis
equations presented with glucose, glutamic acid, and a (1:1) mixture of glucose and glutamic acid as the substrates.

With lactose, there was a reduction in lag and also the time to reach the plateau. The growth patterns were similar to those noticed by other workers in the field\(^{(1)}\). Since lactose is a disaccharide, the higher lag observed for the initial curve compared to that for the monosaccharide, glucose, may be due to hydrolysis requirements. However, with log-phase seeding, the lag was the same for both glucose and lactose, as shown in Figure 4.

With aspartic acid, there was a reduction in lag and also in the time to attain the plateau. The data obtained substantiate the equations developed for synthesis. Similar results are reported by other workers in the field\(^{(20)}\) using pure cultures.

With propionic acid, there was a reduction in lag and also in the time to attain the plateau. The data obtained and the synthesis equations presented are in agreement. Similar results are reported by Barker\(^{(5)}\) using a colorless alga *Prototheca zopfii*.

Although variable lag periods and variable effectiveness of log-seeding were noted with acetic acid, the plateau B.O.D. value was remarkably reproducible. The synthesis equations presented are in approximate agreement with re-
results reported by Barker(5), Giesberger(6) for pure cultures as shown in Table I(10).
CONCLUSIONS

The following conclusions may be drawn.

1) The relation between respiration and synthesis in soluble substrates with mixed microbial populations can be expressed in the form of a stoichiometric equation.

2) The plateau in oxygen utilization is highly reproducible and is characteristic of a specific substrate.

3) The plateau theoretically occurs at 41, 40, 39, 44, 50, 52, and 41 per cent of the theoretical oxygen demand in case of glucose, a (1:1) mixture of glucose and glutamic acid, glutamic acid, aspartic acid, acetate, propionate and lactose respectively, based on stoichiometric equations derived from experimental data.

4) Patterns of growth curves and microbial assimilations are similar to those reported by other workers using pure cultures.

5) The log-phase seeding technique produced a reduction in lag for all of the substrates studied.

6) The log-phase seeding dilution technique reduced the time for attainment of the plateau for all the soluble substrates studied except glucose and a (1:1) mixture of glucose and glutamic acid.
7) At glucose and lactose concentrations higher than 8.06 mg/L, a deficiency in nitrogen requirements exists if the standard dilution technique nutrients are used.


