# THE EFFECT OF INITIAL SUBSTRATE CONCENTRATION UPON EXPONENTIAL OXYGEN UPTAKE RATES

By

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# THE EFFECT OF INITIAL SUBSTRATE CONCENTRATION UPON EXPONENTIAL OXYGEN UPTAKE RATES

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# CHAPTER I

## INTRODUCTION

"The BOD test is paradoxical. It is the basis of all regulatory actions, and is used routinely in almost all control and research studies on sewage and industrial waste treatment. It has been the subject of a tremendous amount of research, yet no one appears to consider it adequately understood or well adapted to his work." Although this statement by Hoover, Jasewicz, and Porges (1) was made in 1953 and much research has been done since then, it is still basically true. In spite of the fact that the test is not well understood, the concept of determining the amount of oxygen and the rate of oxygen uptake of aerobic organotrophic microorganisms utilizing an available carbon source is certainly defensible. Unfortunately, the test does not mirror the concept. For example, one point along the oxygen uptake curve is used to predict the total oxygen uptake and the kinetics of the oxygen uptake curve. Also, the monomolecular law (single phase first order decreasing rate) has been used for the "standard" BOD kinetics even though there are ample data in the literature showing that, under conditions in the BOD test, microbial growth can attain a logarithmic growth phase followed by a declining growth phase, and that the resulting oxygen uptake curve is similar to the growth curve. Furthermore, it is assumed that the same oxygen uptake rate constant will be obtained regardless of

the concentration of the carbon source. However, work accomplished thus far in these laboratories has tended to support the "Monod relationship," which relates the logarithmic growth rate constant to substrate concentration. The Monod relationship yields a rectangular hyperbola when the logarithmic growth rate constant (i.e., specific growth rate) is plotted against substrate concentration.

The study herein reported was designed to determine if the logarithmic oxygen uptake rate constants did follow the Monod relationship, and if so, to what extent the use of the dilution technique for the BOD test affects the rate determined.

# CHAPTER II

## LITERATURE REVIEW

The biochemical oxygen demand (BOD) test has reached its present status through a long history of development. In 1962, O'Brien and Clark (2) published an extensive review of the historical development of the BOD test. Also, reports by Streeter and Phelps (3), and Theriault (4), are good sources on the early development and research on the BOD test.

Two facets which are the subject of controversy regarding the BOD test are: (1) the oxygen uptake is assumed to be approximately defined by first order decreasing rate kinetics, and (2) the oxygen uptake in the dilute BOD bottle system is approximately the same as in less dilute systems which may exist in the stream.

In 1909, Phelps (5) stated: "It is a principle of physical chemistry that the velocity of a chemical reaction is some power of the reacting substances. In the simplest case, the velocity varies directly as the concentration. The bacterial reactions which have been studied also conform to this law, and it has therefore been applied to the present study." Thus, Phelps became the first researcher to propose applying the monomolecular law to oxygen uptake kinetics. Theriault (4) supported Phelps' proposed monomolecular law. He also developed the first order concept into the mathematical form in use today, and

proposed the rate constant, $K_1 = 0.1\ day^{-1}$. Later, this constant was widely accepted as being applicable to any municipal sewage. It should be pointed out that both Phelps' and Theriault's results were based on studies of polluted river water. Phelps later stated: "There is in fact, no real justification for holding that the rate of decrease of BOD is exactly monomolecular...there are some facts suggesting that the monomolecular law does not exactly express the situation" (6).

In fact, an examination of the literature reveals that there were many cases for which the monomolecular relationship was not observed. Due to efforts of various researchers to elucidate the causes for departures from first order decreasing kinetics, a large amount of criticism of the "monomolecular law" has built up over the years. While attempting to develop a short term total oxygen demand test, Busch, Grady, Rao, and Swilley (7) concluded from their data and from the slope of the short term BOD curve that first order rate kinetics should not be employed to describe $O_2$ uptake. Gates, Mancy, Shafie, and Pohland (8) found that the monomolecular expression for oxygen uptake was invalid for experiments utilizing natural substrates and heterogeneous cultures, pure substrates and pure cultures, and pure substrates and dominate cultures. Similar results had been reported by Bhatla and Gaudy (9), and Gunnerson (10).

In 1911, Muller (4) observed that the rate of deoxygenation appeared to be closely related to the rate of bacterial increase. In addition to this, Theriault (4) concluded from Muller's results that when the bacteria were in a state of multiplication, the oxygen uptake rate was about ten times higher than when the bacteria count was stationary. Similar results were found by Purdy and Butterfield (11),

Butterfield (12), and McWhorter and Heukelekian (13).

Hoover, Jasewicz, and Porges (1) found that BOD exertion on a milk waste was exerted in two distinct phases. First there was a rapid growth of cells with assimilation of available nutrients into cells, followed by the subsequent slow endogenous respiration of these cells. The first phase was completed within twenty-four hours. In addition to this, the rate constant of the endogenous phase was suggested to be the same as that proposed by Theriault (4) ($K_1$ = 0.1 $day^{-1}$). It should be noted that an actively growing inoculum with a high ratio of bacterial solids to substrate was used.

Also, Buswell, Muller, and Van Meter (14) observed that the BOD exertion curve for an ammonia substrate with Nitrosomonas for seed was dependent upon the rate of multiplication of organisms rather than the concentration of substrate. Buswell, et al. found no evidence of a monomolecular relationship between the rate of oxygen uptake and the substrate concentration.

While developing a short term BOD test in 1958, Busch (15) observed that for soluble substrates, the progression of BOD exertion by mixed cultures of sewage organisms was a multi-stage reaction. He noticed two phases of oxygen uptake, separated by a plateau. The first stage was attributed to the conversion of available substrate into cell material and intermediate storage products. The second phase was reported to be caused by endogenous respiration of the bacteria and the action of predators, e.g., protozoa feeding on bacteria.

More recently, much work has been done in the bioengineering laboratories at Oklahoma State University on the occurrence and causation of diphasic oxygen uptake. Gaudy, Bhatla, and Abu-Niaaj (16),

Gaudy and Bhatla (17), and Follett and Gaudy (18) found from their studies that diphasic oxygen uptake occurred regardless of the initial substrate concentration and the type and amount of initial seed inoculum, so long as cell multiplication occurred. In all of these studies, the first stage was associated with an increasing bacterial count. Also, the discontinuity in kinetics always occurred in the range of maximum population density. The second phase was accompanied by a decrease in viable count.

The general occurrence of diphasic oxygen uptake has been demonstrated and observed in BOD progressions, as evidenced in the literature cited. Also in all cases cited, the first phase was related to a rapid increase in bacterial count.

The rapid first stage oxygen uptake has been observed by many researchers. Orford and Ingram (19) conducted experiments concerning the deoxygenation of sewage. They noted that there was no fundamental biological reason why oxidation should take place according to the monomolecular oxidation reaction. From their results they concluded: (1) The monomolecular equation is a poor expression for analysis of biological oxidation, because the two parameters of the equation, $K_1$ and $L_o$, are not constants. (2) Due to the variation of $K_1$ and $L_o$ with time of observation, the parameters have very little biological significance as measures of oxidation speed or strength. (3) "The logarithmic equation has more biological significance and is better adapted to the interpretation of biological oxidation phenomena than the monomolecular equation." It should be noted that the method Orford and Ingram proposed involved plotting oxygen uptake vs. logarithmic time.

In 1963, Gaudy, Bhatla, and Abu-Niaag (16) stated the following

concerning the first phase of oxygen uptake: "The increasing portion of the curve may often be shown to be first order, increasing rate. With an acclimated seed, this type of kinetics would be expected if the system was started using a low initial inoculum. The upswing portion would then correspond to a log growth phase and not a lag phase." Later, Follett and Gaudy (18) noted that at initial seed populations low enough to allow an increase in cell numbers, the first phase of oxygen uptake generally corresponded to a log growth phase of cell multiplication. Using information obtained by plotting their oxygen uptake data on semi-log paper (logarithmic accumulated $O_2$ uptake vs. time), they indicated that the first part of the curve may be described as a first order increasing rate curve which corresponded to bacterial logarithmic growth.

Also, Follett and Gaudy (18) pointed out that there was a dependence of the oxygen uptake rate upon substrate concentration during the first phase of oxygen uptake, but no explicit relationship was observed. However, Theriault (4) had stated: "By the use of more favorable forms of analytical procedure, it can now be more readily demonstrated that with a variety of organic waste, the rate of oxidation is not affected by dilution."

In addition to Follett and Gaudy, several researchers have observed results in opposition to Theriault's conclusion. Caldwell and Langelier (20) observed an increase in the rate of oxidation when undiluted samples were used. Later, Heukelekian and Gellman (21) found that the oxygen demand values obtained using a Warburg respirometer was higher in the undiluted samples. Furthermore, Orford and Ingram (19) noted that the biological oxidation rate was governed by the relative

proportions of the various types of material to be oxidized.

In 1949, Monod (22) suggested an empirical expression which related the specific growth rate of bacteria to substrate concentration. The equation yields a rectangular hyperbola when the specific growth rate (also the exponential growth rate constant) is plotted against substrate concentration. This curve becomes asymptotic to the maximum non-substrate limited growth rate attainable by that particular system. In 1967, Gaudy, Ramanathan, and Rao (23), using heterogeneous populations in completely mixed reactors, found that "in general, the data substantiated the single phase relationship between growth rate and substrate concentration described by the Monod equation, $\mu = \mu_{max} S / K_s + S$, where $\mu$ is the logarithmic growth rate constant, S is the substrate concentration, $\mu_{max}$ is the maximum logarithmic growth rate constant for the culture under the conditions used, and $K_s$ is the substrate concentration at which $\mu = \mu_{max/2}$. In 1970, Peil and Gaudy (24) demonstrated that the Monod relationship could be used to describe growth in heterogeneous populations metabolizing a variety of substrates, including concentrated municipal sewage.

As has been previously stated, the oxygen uptake tends to parallel the bacterial growth curve during the first phase of oxygen uptake. Thus, it could be anticipated that some relationship might exist between the logarithmic oxygen uptake rate and the initial substrate concentration. Jennelle and Gaudy (25), after investigating oxygen uptake rates at various concentrations of substrate, stated: "Oxygen uptake rate constants were observed to increase with increasing concentration of carbon source, thus militating against direct use of the usual dilution technique for predicting rate of deoxygenation in receiving

streams. The relationship between specific $O_2$ uptake rate and substrate concentration approximated a hyperbolic function similar to the Monod relationship for specific growth rate and substrate concentration." Since two experiments were run, Jennelle (26) suggested that additional research was warranted to further substantiate this proposal.

# CHAPTER III

## MATERIALS AND METHODS

### I. Indirect Method of Measuring $O_2$ Uptake

#### A. Laboratory Equipment

Three pyrex battery jars (8 inches in diameter and 18 inches deep) were used as aeration vessels. The experiments were conducted at $30^{o}C$ using a 36" x 13" x 15" water bath with a Precision Scientific Lo-temptrol temperature control unit to maintain a constant temperature. Mixing was accomplished by three Sargent magnetic stirrers. A stop watch was employed to synchronize the rates of the stirring bars. A Precision Scientific galvanic cell oxygen analyzer was used to measure the dissolved oxygen (DO).

#### B. Composition of the Synthetic Waste

The following volumes of inorganic salts were added for each ml of 100,000 mg/l glucose stock solution added to the vessels:

1) 1 ml $(NH_4)_2SO_4$
2) 1 ml $MgSO_4 \cdot 7H_2O$
3) 1 ml $FeCl_3 \cdot 6H_2O$
4) 1 ml $MnSO_4 \cdot H_2O$
5) 1 ml $CaCl_2$
6) 1 ml potassium phosphate buffer

These solutions were prepared in accordance with Standard Methods (27).

C. Source of Microbial Population

The seed was grown up in a standard batch tube from an initial inoculum of municipal sewage seed obtained from the primary clarifier of the Stillwater municipal waste treatment plant. Before the unit was fed each day, 1/3rd of the mixed liquor was wasted. Then the cells were allowed to settle for an hour, and 1/2 of the remaining volume was wasted. The batch unit was fed glucose stock solution and appropriate amounts of the inorganic salt solutions diluted so that the final concentration of glucose was 1000 mg/l. After the substrate was added, the volume of water was adjusted to the original volume, using distilled water and 10 ml/l of the total volume of tap water. The seed to be used in an experiment was harvested from the supernatant after allowing the cells in the batch tube to settle for five minutes. The seed was harvested just before the unit was fed in all cases.

D. Experimental Procedures and Determination of Reaction Constants

1. Reaeration

At the start of each experiment, the pyrex battery jars were washed, rinsed, and filled with about twelve liters of tap water. Then the vessels were set in the water bath over night to allow the water to reach a temperature of $30^{o}C$, and the magnetic stirrers were turned on to aid the system to reach equilibrium.

Throughout this investigation, the dissolved oxygen reagents used were those prescribed in Standard Methods for Examination of Water and Wastewater (27) for the Alsterberg modification of the Winkler method.

These reagents were used to determine the DO of the reaction liquor, and this determination was used to standardize the galvanic cell oxygen analyzer. For all of the DO readings, the probe was inserted downward to within approximately one inch of the bottom of the reactor.

After standardization of the probe, the volume of the reaction liquor was decreased to ten liters. Sufficient sodium sulfite to remove 7.5 mg/l of DO was added to remove the DO; 0.02 mg/l cobalt chloride catalyst was also added. Using the probe, DO was measured every fifteen minutes until the zero time point was established. Readings were taken at various times during the next twenty-four hours, at which time 300 ml of the reaction liquor was removed to re-standardize the probe, and the experiment was continued.

Before the reaeration constant, $K_2$, could be found, the correct saturation DO (Cs) has to be determined. Cs was estimated using the $\alpha$-method, which has been outlined by Isaacs and Gaudy (28). Once Cs was found, the log of the $O_2$ deficit vs. time was plotted. The slope of the straight line drawn through the points gave the reaeration constant, $K_2$.

2. Deoxygenation

Before the seed and the substrate were added to the vessels, the turning rates of the magnetic stirrers were checked to ensure that they were turning at the same rate as at the start of the experiment.

Next, the appropriate amounts of glucose stock solution and inorganic salts were added to the reaction vessels to obtain the desired substrate levels. After the substrate was added, 25 ml of seed were added to each vessel; then enough water was added to adjust the volume of reaction liquor to ten liters. The DO was monitored with

the probe until it had started back up from the bottom of the DO sag curve, at which time the standardization of the probe was checked, and the experiment was ended.

The $O_2$ uptake rate was calculated using the following procedure:

1. Let $D_1$ and $D_2$ be the deficits for times $t_1$ and $t_2$, respectively.
2. Let $DO_1$ and $DO_2$ be the dissolved oxygen levels at times $t_1$ and $t_2$.
3. $O_2$ uptake between times $t_1$ and $t_2$ is

$$\frac{K_2D_1 + K_2D_2}{2}\left(t_2 - t_1\right) - \left(DO_2 - DO_1\right)$$

4. The sum of the $O_2$ uptakes over all of the time periods gave the total $O_2$ uptake.

The $O_2$ uptake rate, $\mu_{O_2}$, was found by measuring the slope of the straight line portion of the curve when the logarithm of accumulated $O_2$ uptake vs. time was plotted.

## II. Direct Measurement of $O_2$ Uptake Using the Warburg Apparatus

### A. Composition of Synthetic Waste

The following substrate was used as the stock solution for experiments 5-8, 12-14:

| | | |
|---|---|---|
| Glucose | 1000 | mg/l |
| $(NH_4)_2 \cdot SO_4$ | 500 | mg/l |
| $MgSO_4 \cdot 7H_2O$ | 100 | mg/l |
| $FeCl_3 \cdot 6H_2O$ | .5 | mg/l |
| $CaCl_2$ | 7.5 | mg/l |
| $MnSO_4 \cdot H_2O$ | 10 | mg/l |
| Potassium phosphate buffer, 1.0 m | 10 | ml/l |
| Tap water | 100 | ml/l |

Appropriate amounts of stock solution were used and diluted with distilled water to the desired concentrations.

The following substrate was used as the stock solution in experiments 9-11:

| | | |
|---|---|---|
| Acetic acid | 1000 | mg/l |
| Potassium phosphate buffer 1.0 m | 55 | ml/l |
| $(NH_4)_2\ SO_4$ | 500 | mg/l |
| $MgSO_4 \cdot 7H_2O$ | 100 | mg/l |
| $FeCl_3 \cdot 6H_2O$ | 0.5 | mg/l |
| $MnSO_4 \cdot H_2O$ | 10 | mg/l |
| $CaCl_2$ | 7.5 | mg/l |
| Tap water | 100 | ml/l |

Once again, appropriate amounts of stock solution were used and diluted with distilled water to the desired concentration.

B. Source of Microbial Population

For experiments 5-8, heterogeneous microbial cultures were developed. The initial inoculum of municipal sewage seed was obtained from the primary clarifier effluent of the Stillwater municipal waste treatment plant. The cultures were grown in a 250-ml Erlenmeyer flask and aerated on a shaker apparatus (Eberbach) operating at 120 opm. The initial reaction liquor consisted of 45 ml of stock substrate solution and a 5-ml inoculum of sewage seed. After twenty-four hours, five ml of the mixed liquor was transferred to a flask containing 45 ml of stock substrate. After twelve hours, the mixed liquor was used as seed.

For experiments 9-11, heterogeneous microbial cultures were developed. The initial inoculum of municipal sewage seed was obtained from the primary clarifier effluent of the Stillwater municipal waste treatment plant. The cultures were developed in a 500-ml Erlenmeyer flask

and aerated on a shaker apparatus (Eberbach) operating at 100 opm. The initial reaction liquor consisted of 95 ml of stock acetic acid substrate and a 5-ml inoculum of sewage seed. The seed was transferred two times before use.

For experiments 12-14, Flavobacterium sp. was used for seed. During the investigation, the organism was maintained on Difco nutrient agar slants and an inoculum from a fresh slant was grown through two transfers on glucose minimal salts medium prior to use in an experiment.

In all Warburg experiments, enough seed was added to the Warburg flask to yield optical density of approximately 0.045 on a Bausch-Lomb Spectronic 20 spectrophotometer/colorimeter at a wave length of 540 nm, using a distilled water blank.

C. Experimental Procedure

The acclimated cells were used to inoculate 140-ml Warburg flasks containing appropriate amounts of stock substrate solution. The reaction liquor volume in the flasks was adjusted to 40 ml by adding distilled water. Also, 1.5 ml of 20 percent KOH was placed in the center well. The Warburg respirometer was operated at $25^{o} \pm 0.5C$ with a shaker flask rate of 110 opm. The samples were allowed to equilibrate for fifteen minutes before closing the manometers. Readings were taken at short intervals (usually every fifteen minutes) in order to closely define the $O_2$ uptake curve. Readings were taken until an adequate number of points had been obtained to define a logarithmic phase. Accumulated oxygen uptake (logarithmic) vs. time was plotted on semi-logarithmic paper, and the uptake rates were obtained by taking the slope of the resulting line.

## III. Oxygen Uptake as Related to Growth

### A. Synthetic Waste and Source of Microbial Population

The glucose minimal salts medium used for the Warburg studies was used for these experiments. The Flavobacterium sp. used in Warburg experiments 12-14 was employed in this experiment. This population was also developed in the same method described in section IIB, this Chapter.

### B. Experimental Procedure

The procedure for experiments employing the Warburg apparatus was the same as described above (see oxygen uptake using the Warburg apparatus). For the growth rate studies, 250-ml Erlenmeyer flasks containing various concentrations of substrate were inoculated with enough seed to produce an optical density of approximately 0.032. The Erlenmeyer flasks were then aerated on an Eberbach shaker at 110 opm. The growth was determined by measuring the optical density, using a Bausch-Lomb Spectrophotometer at a wave length of 540 nm. Readings were taken at 15-minute intervals, and the growth rates were determined from the straight line portion of a semi-logarithmic plot of optical density vs. time.

## IV. Direct Measurement of $O_2$ Uptake Using BOD Bottles

### A. Synthetic Waste and Seed

The medium used for the indirect method of measuring $O_2$ uptake was used for these experiments. The same Flavobacterium sp. used in Warburg experiments 12-14 was used for seed. The same procedure was used to prepare the seed. However, various amounts of seed were used, as indicated in Chapter IV.

B. Experimental Procedure

Dilution water was prepared in a 25-liter aspirator bottle. Distilled water was used as dilution water and the seed was added directly to the dilution water before the water was oxygenated. Compressed oxygen was applied to the dilution water through two glass diffusers for about three minutes; the resulting DO was approximately 30 mg/l. Appropriate amounts of stock medium were added to the BOD bottles just prior to filling the BOD bottles with dilution water. Usually four different concentrations of substrate were used.

A 3/8ths-inch diameter latex hose was attached to the spigot of the aspirator bottle. The BOD bottles were filled and capped as rapidly as possible so as to give uniform conditions in all bottles in the system. The first and the last bottles filled from each substrate concentration were analyzed for the initial DO, and good agreement was found.

The bottles were incubated at ambient temperature ($23 \pm 1^{o}C$). DO determinations were performed at intervals of about two hours. The DO was determined using the reagents prescribed in Standard Methods for Examination of Water and Wastewater (27) for the Alsterberg modification of the Winkler method.

# CHAPTER IV

# RESULTS

## I. General Comments

These experiments were designed to evaluate the effect of dilution upon the rate of biological oxidation.

The symbols used in presentation of results are as follows:

| | |
|---|---|
| $\mu_{O_2}$ | the logarithmic (exponential) oxygen uptake constant, also referred to as specific $O_2$ uptake rate |
| $S_o$ | the initial substrate concentration |
| $\mu_{O_2 max}$ | the maximum logarithmic oxygen uptake rate for the culture for the conditions used |
| $K_s'$ | the substrate concentration at which $\mu_{O_2} = \frac{\mu_{O_2 max}}{2}$ |
| $\mu$ | the logarithmic (exponential) growth rate constant, also referred to as specific growth rate |
| $\mu_{max}$ | the maximum logarithmic growth rate constant rate for the culture for the conditions used (natural logarithmic) |
| $K_s$ | the substrate concentration at which $\mu = \frac{\mu_{max}}{2}$ |

## II. Oxygen Uptake in Open Systems (Battery Jars)

Figure 1 shows the course of the oxygen uptake curve and the sag curve for a system in which the initial concentration of glucose was 50 mg/l. The results from this system are indicative of results obtained throughout these experiments. As can be observed from the oxygen uptake curve on Figure 1, the monomolecular relationship was not

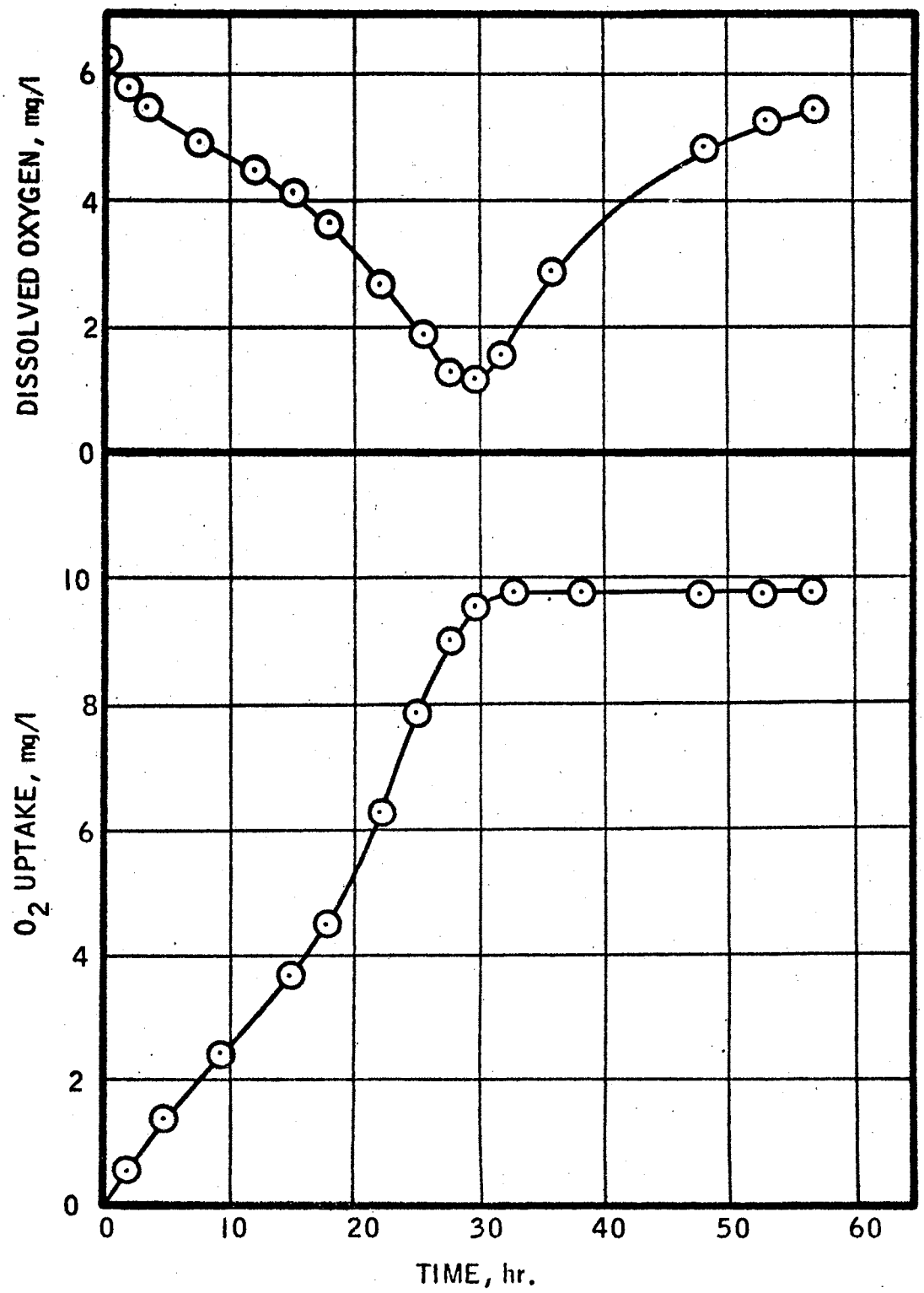



Figure 1. Oxygen Sag and Accumulated $O_2$ Uptake Curves for Experiment 4, $S_o$ = 50 mg/l.

observed in this experiment, nor was it observed in any of the experiments performed. In fact, a logarithmic uptake phase was observed in all cases. Figure 2 is a semi-logarithmic plot of the oxygen uptake data of Figure 1. As can be seen from this plot, there was a definite logarithmic phase between hours 7.5 and 27.5. The data obtained from this experiment as well as from those of others accomplished using the open battery jar technique, are tabulated in Table I.

Although logarithmic phases were observed in all experiments, not enough experiments were run to determine a relationship between substrate concentration and the logarithmic oxygen uptake rate ($\mu_{O_2}$). There was, however, some indication, particularly in experiments 1 and 2, that $\mu_{O_2}$ tended to increase as $S_o$ was increased (i.e., as dilution factor was decreased).

TABLE I

SUMMARY OF RESULTS OF EXPERIMENTS IN OPEN REACTORS
(Experiments 1-4)

| Exp. # | Substrate Concentration mg/l | Time, hr. End of Log | Time, hr. Bottom of Sag | Maximum Deficit mg/l | $\mu_{O_2}$ $hr^{-1}$ |
|---|---|---|---|---|---|
| 1 | 10 | 21 | 21 | 2.2 | .019 |
| 1 | 20 | 25 | 25 | 4.4 | .042 |
| 1 | 30 | 26 | 29 | 4.9 | .064 |
| 2 | 40 | 50 | 52 | 5.9 | .017 |
| 2 | 50 | 55 | 55 | 6.3 | .025 |
| 2 | 60 | 50 | 45 | 6.2 | .0218 |
| 3 | 20 | 50 | 50 | 2.7 | .02 |
| 3 | 30 | 69 | 72 | 4.7 | .015 |
| 4 | 25 | 22 | 22 | 3.7 | .033 |
| 4 | 50 | 27.5 | 28 | 5.7 | .0335 |
| 4 | 75 | 28 | 27 | 6.3 | .033 |

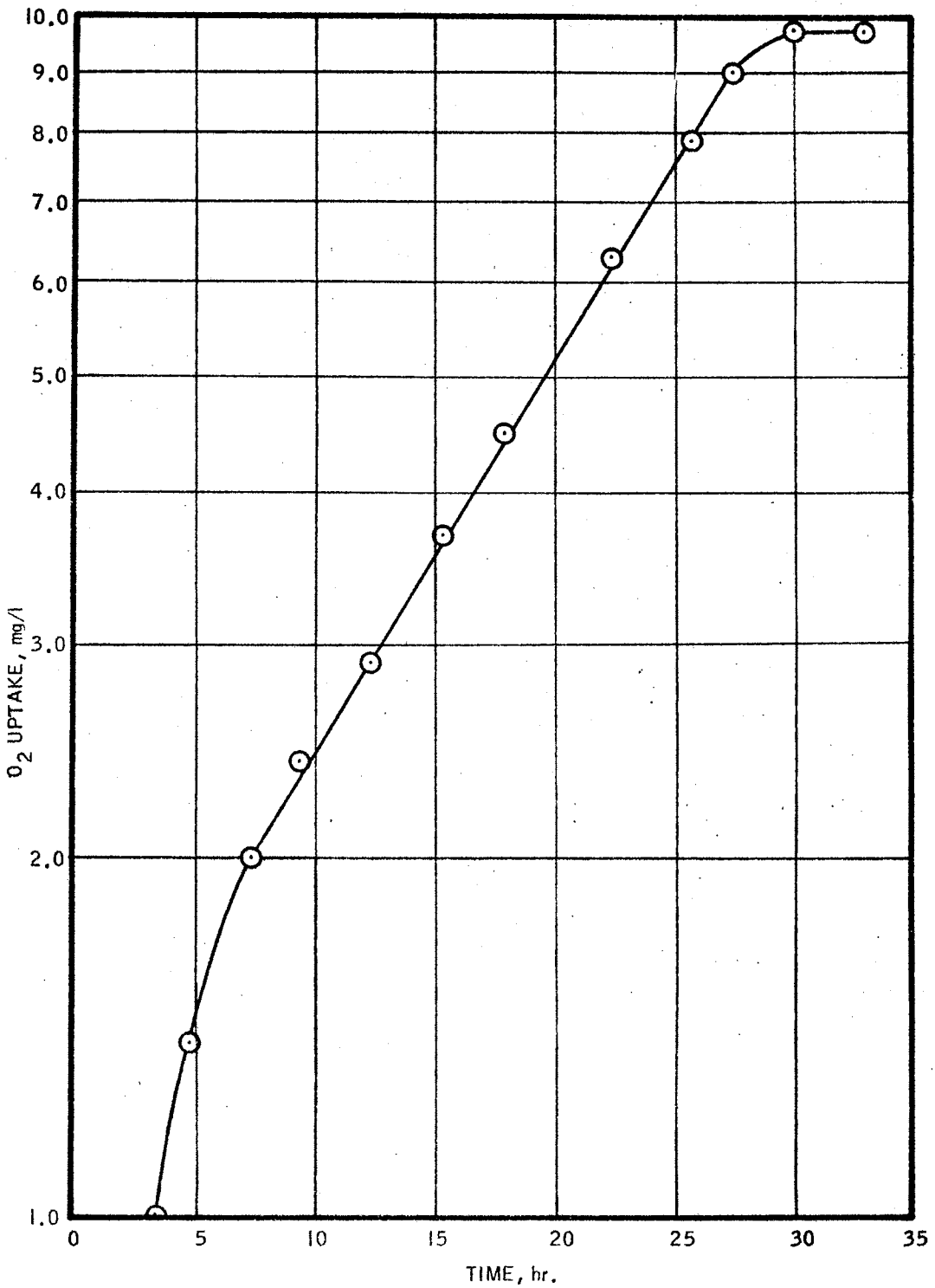



Figure 2. Logarithmic $O_2$ Uptake vs. Time for Experiment 4, $S_o$ = 50 mg/l.

In all of the experiments, there was a very close correlation between the end of the logarithmic oxygen uptake phase and the bottom of the sag curve. In all but one of the experiments, the two events occurred within three hours of one another. This result indicates that the logarithmic phase might be a major factor in determining when the critical deficit will occur and, if this is so, the magnitude of $\mu_{O_2}$ would exert an influence on the magnitude of the critical deficit. Due to the failure to determine a definite relation between substrate concentration and specific oxygen uptake rates using the open jar technique, and due to the apparent importance of the oxygen uptake rates, further experiments were run to help determine this relationship using other techniques to measure accumulated oxygen uptake.

## III. Oxygen Uptake in the Warburg Apparatus

These experiments were conducted using either glucose or acetic acid for a substrate with either an acclimated heterogeneous culture or a pure culture of *Flavobacterium sp.* for seed. The experiments were grouped in the following order: a) glucose minimal salts medium with heterogeneous population for seed, b) acetic acid substrate with heterogeneous population for seed, and c) glucose minimal salts medium with *Flavobacterium sp.* for seed.

### A. Glucose Minimal Salts Medium With Heterogeneous Population for Seed

Figures 3 through 6 show the results for experiments conducted in this phase of the study. These are experiments 5 through 8 of the overall study. In all figures, with the exception of Figure 4, the higher substrate concentrations exhibited the same $\mu_{O_2}$ values as the highest concentration plotted, and their oxygen uptake curves followed

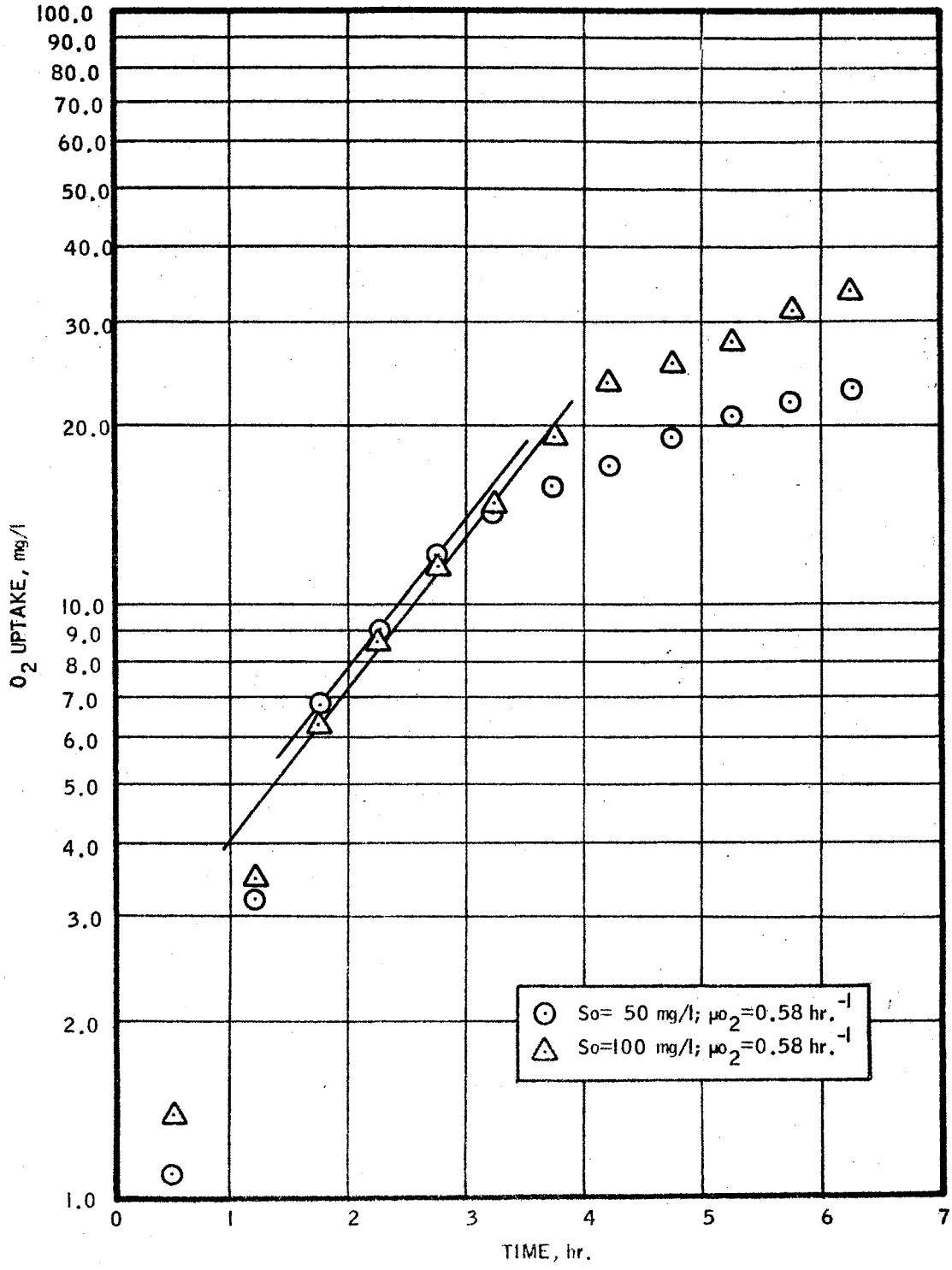



Figure 3. Logarithmic $O_2$ Uptake vs. Time for Experiment 5.

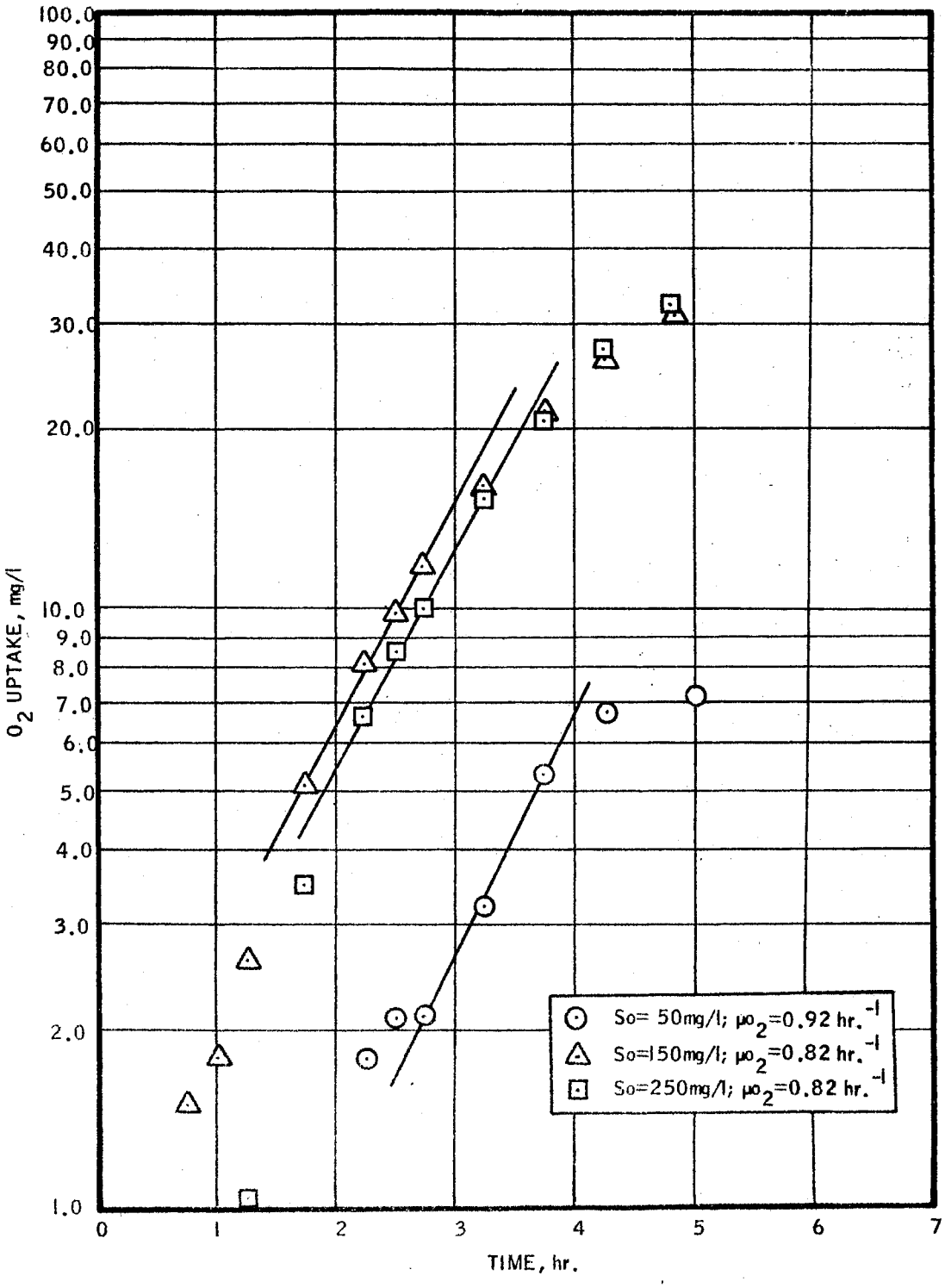



Figure 4. Logarithmic $O_2$ Uptake vs. Time for Experiment 6.

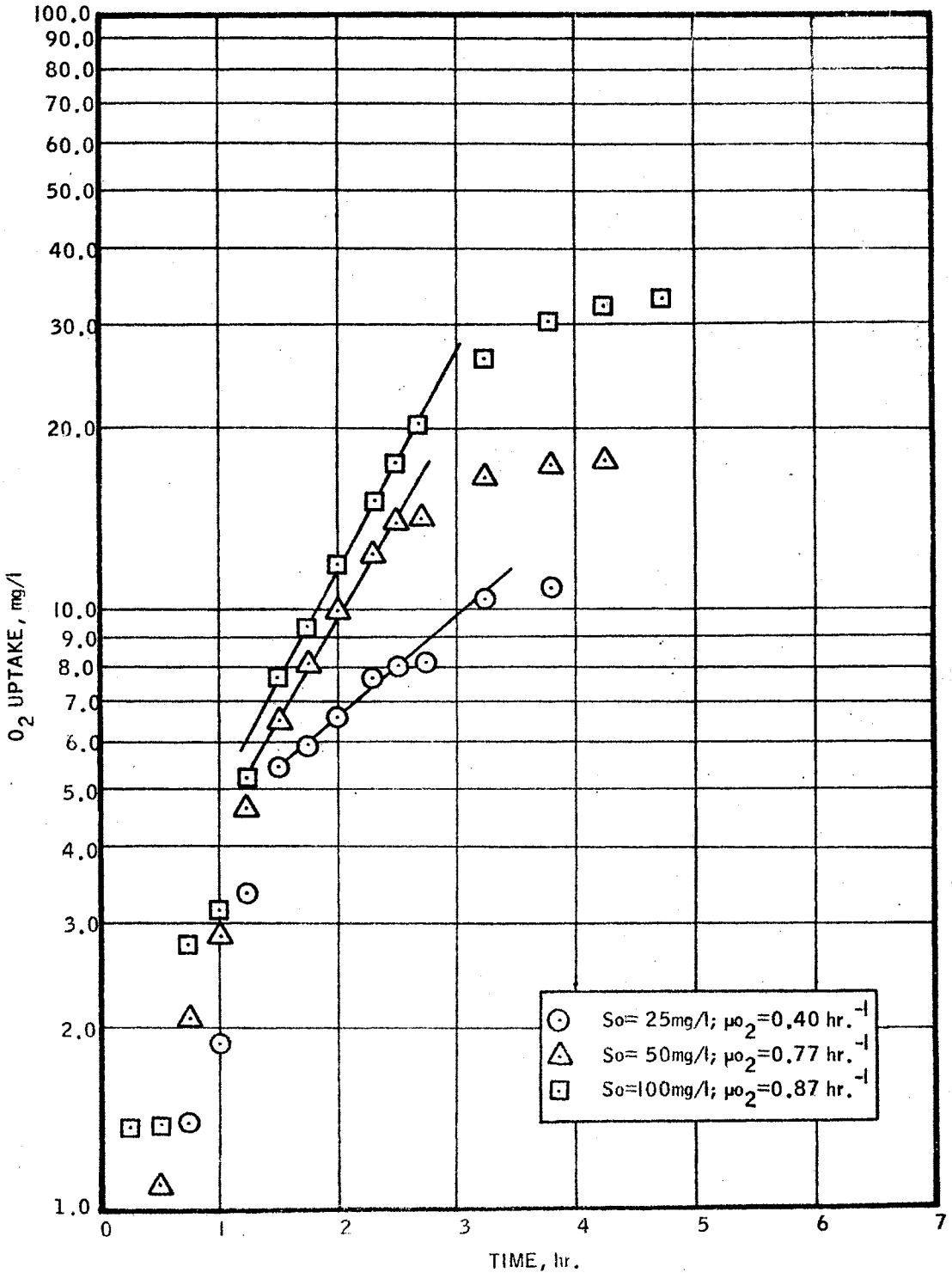



Figure 5. Logarithmic $O_2$ Uptake vs. Time for Experiment 7.

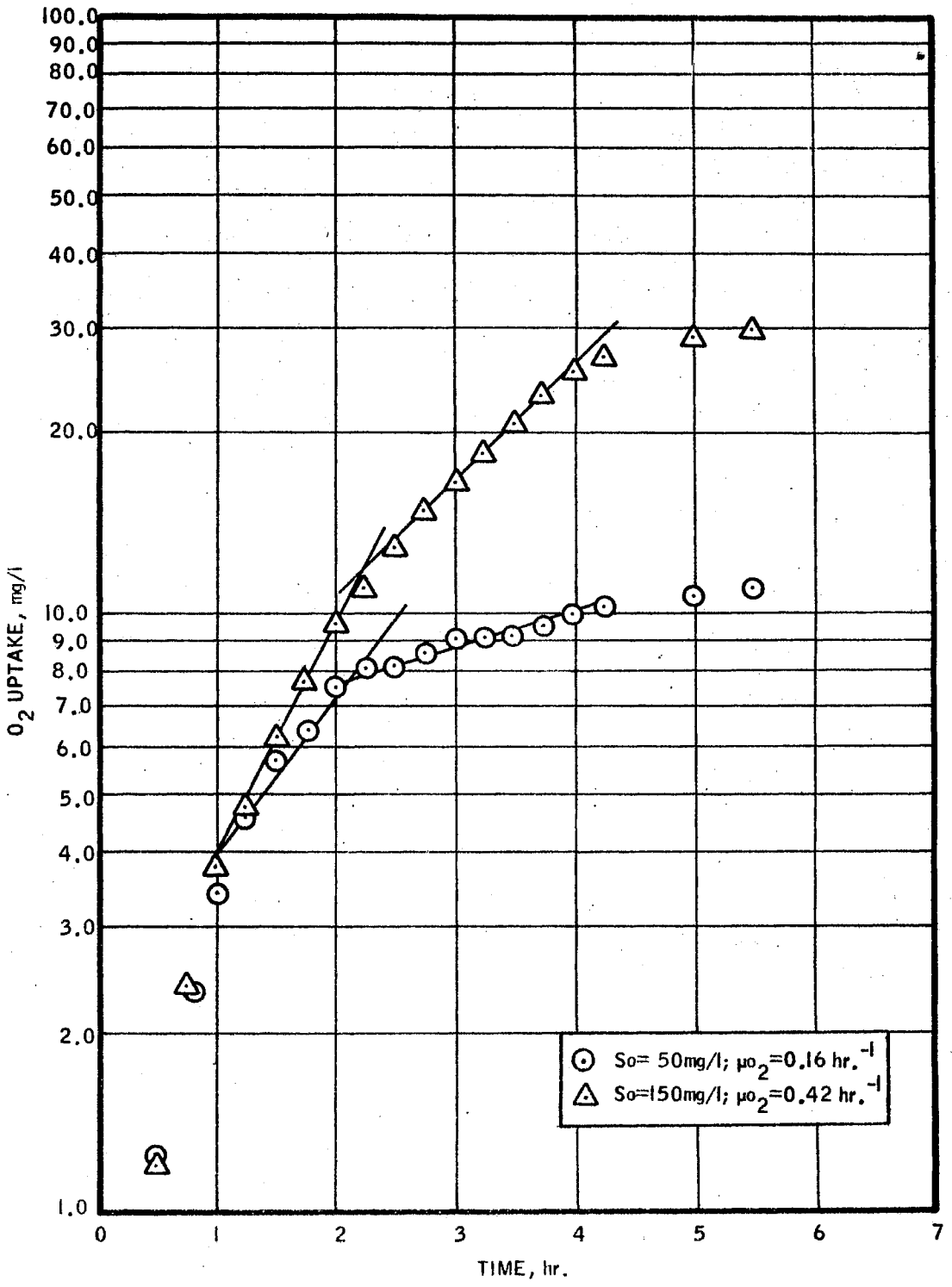



Figure 6. Logarithmic $O_2$ Uptake vs. Time for Experiment 8.

the same course for the highest concentration shown. The results of these experiments are tabulated in Table II.

TABLE II

SUMMARY OF RESULTS FOR $O_2$ UPTAKE RATES USING THE WARBURG RESPIROMETER (Experiments 5-14)

| Exp. # | S (mg/l) | $\mu_{O_2}$ ($hr^{-1}$) | Accumulated $O_2$ Uptake (mg/l)* | Exp. # | S (mg/l) | $\mu_{O_2}$ ($hr^{-1}$) | Accumulated $O_2$ Uptake (mg/l)* |
|---|---|---|---|---|---|---|---|
| 5 | 50 | 0.58 | - | 10 | 60 | 1.03 | 32 |
| | 100 | 0.58 | - | | 120 | 1.17 | 62 |
| | 150 | 0.58 | - | | 250 | 1.26 | 115 |
| | 200 | 0.58 | - | | 400 | 1.26 | 200 |
| | 250 | 0.58 | - | | 1000 | .85 | - |
| 6 | 50 | .92 | - | 11 | 120 | .88 | 60 |
| | 150 | .82 | - | | 250 | 1.00 | 100 |
| | 250 | .82 | - | | 400 | 1.11 | 200 |
| | | | | | 100 | 0.88 | - |
| 7 | 25 | .4 | - | | | | |
| | 50 | .77 | - | 12 | 75 | .39 | - |
| | 100 | .87 | - | | 125 | .42 | - |
| | 125 | .87 | - | | 250 | .46 | - |
| | 150 | .87 | - | | 500 | .51 | - |
| | 200 | .87 | - | | 750 | .54 | - |
| | 250 | .87 | - | | | | |
| | | | | 13 | 50 | .31 | - |
| 8 | 50 | .16 | - | | 125 | .39 | - |
| | 150 | .42 | - | | 250 | .42 | - |
| | 250 | .47 | - | | 375 | .48 | - |
| | 500 | .47 | - | | 750 | .48 | - |
| | 750 | .47 | - | | | | |
| | | | | 14 | 62 | .38 | - |
| 9 | 50 | .8 | 23 | | 125 | .41 | - |
| | 88 | 1.0 | 36 | | 250 | .46 | - |
| | 250 | 1.13 | - | | 500 | .53 | - |
| | | | | | 925 | .56 | - |

*Experiments 5, 6, 7, 8, 12, 13, and 14 were not continued long enough to measure total accumulated $O_2$ uptake.

In all experiments and at all substrate concentrations used (except for experiment 6, at 50 mg/l), the oxygen uptake curve displayed an increasing rate of oxygen uptake which was found to be defined by increasing first-order kinetics when plotted on semi-logarithmic paper. When the data were plotted on semi-logarithmic paper, (logarithmic $O_2$ uptake vs. time), the oxygen uptake curve showed a constantly decreasing slope into the logarithmic phase, which was defined by the straight line portion of the curve. After the logarithmic phase was over, the curve continued to bend over. However, it was observed in experiment 8 that one could approximate an early phase with a straight line; thus it appeared that there were possibly two logarithmic phases. The first "logarithmic phase" did, however, appear to be more related to the early decreasing rate phenomena associated with the other experiments in this series, and the second phase was used to determine the $\mu_{O_2}$ values for this experiment.

Figure 7 shows a plot of $\mu_{O_2}$ vs. $S_o$ for experiment 7 (see Figure 5). This figure is indicative of the results of the other experiments, with the possible exception of experiment 6, which had an unusual oxygen uptake curve at a substrate concentration of 50 mg/l. From Figure 7 it can be seen that $\mu_{O_2 max}$ was achieved at a relatively low substrate level. Only when $S_o$ was below 100 mg/l did $\mu_{O_2}$ show any change with dilution; above this concentration, all concentrations exhibited the same $\mu_{O_2}$. Thus, it appeared that at low substrate concentrations, dilution did affect $\mu_{O_2}$, but there were not enough data at lower concentrations to ascertain what the relation between $\mu_{O_2}$ and $S_o$ was.

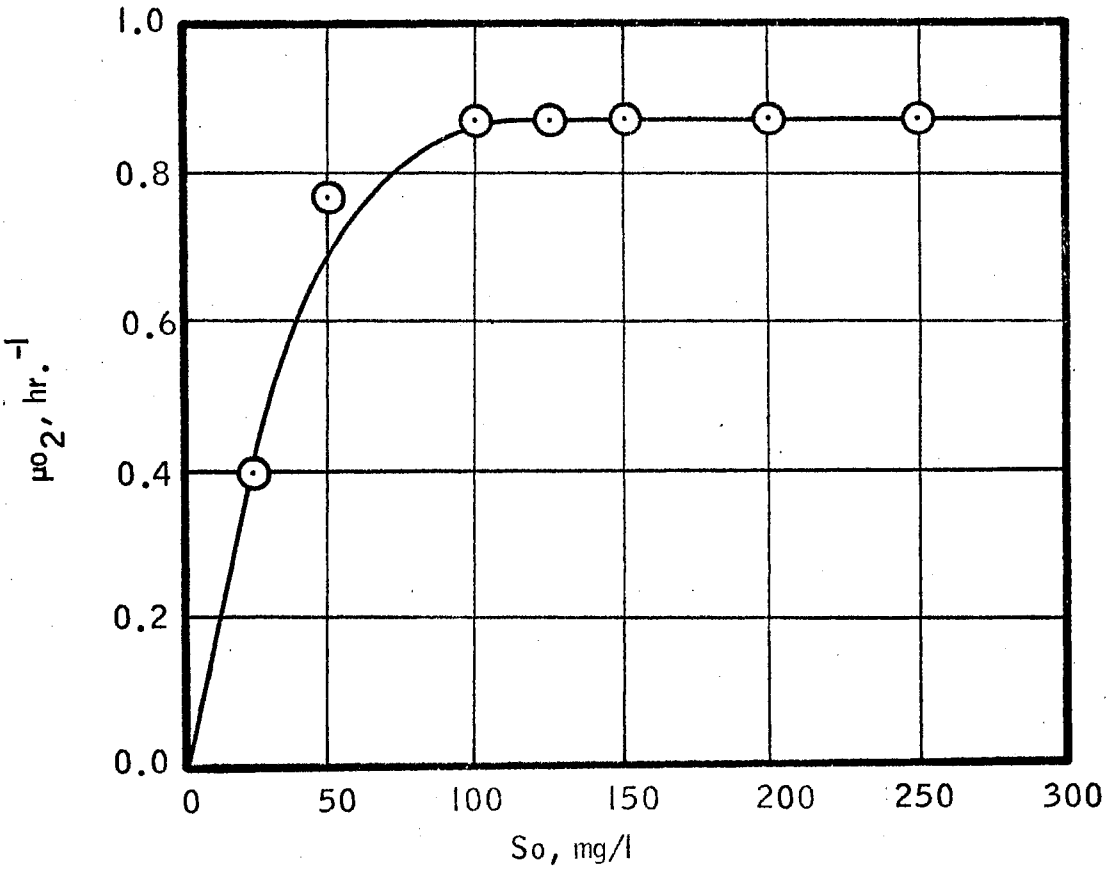



Figure 7. $\mu_{O_2}$ vs. $S_o$ for Experiment 7.

B. Acetic Acid as Substrate With a Heterogeneous Microbial Population for Seed

Figures 8 and 9 are semi-logarithmic plots of $O_2$ uptake vs. $S_o$ for experiment number 10. The other experiments run using acetic acid for a substrate closely follow the course of experiment number 10, so only those two figures are shown. The data from experiments 9 and 11 are tabulated in Table II.

In these three experiments, the oxygen uptake curve displayed an increasing rate which was found to be described by increasing first-order kinetics. When the data were plotted on semi-logarithmic paper, the oxygen uptake curve once again showed a constantly decreasing slope into the logarithmic phase. With the exception of $\mu_{O_2}$ at 1000 mg/l in experiments 10 and 11, the $\mu_{O_2}$ values increased with an increase in substrate, but no exact relationship could be determined. The lower $\mu_{O_2}$ values at 1000 mg/l correspond to findings by Goldstein (29). In studying specific growth rates of heterogeneous microbial populations on the same acetic acid substrate used in these experiments, Goldstein found $\mu$ at 1000 mg/l was lower than $\mu$ at 400 mg/l in some cases.

It is interesting to note the rapid rates of oxygen uptake and the relatively large amounts of oxygen used in these experiments. In experiment 10, which was continued until the oxygen uptake curve approached an upper limit, slightly over one-half of the theoretical oxygen demand was utilized by the microbial population. This would indicate relatively lower sludge yield on this substrate than on carbohydrate, a finding that is in substantial agreement with that of Goldstein (29).

C. Glucose Substrate With *Flavobacterium* sp.

As noted earlier, the oxygen uptake rate appeared to be affected

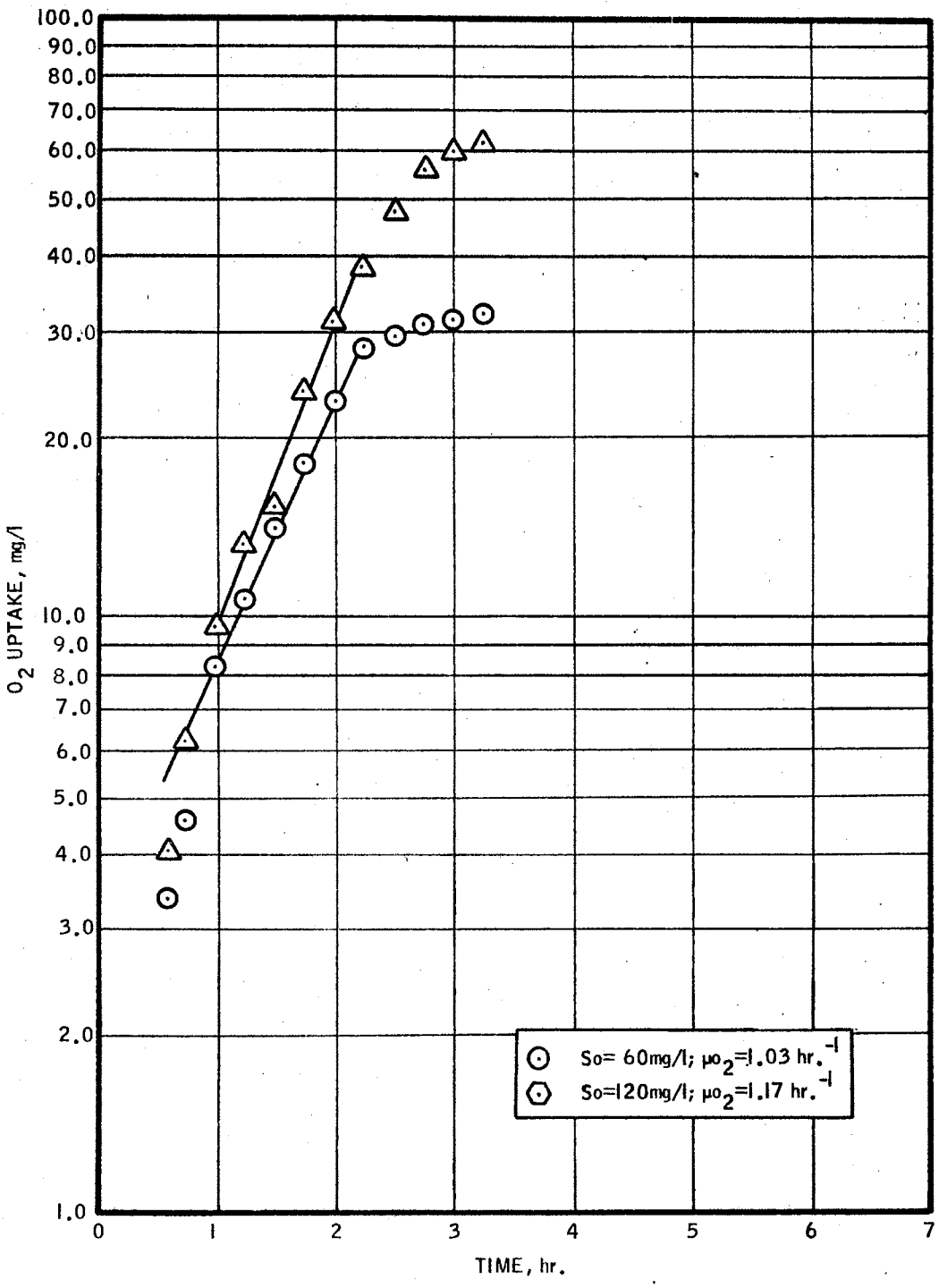



Figure 8. Logarithmic $O_2$ Uptake vs. Time for Experiment 10, With $S_o$ = 60 mg/l and $S_o$ = 120 mg/l.

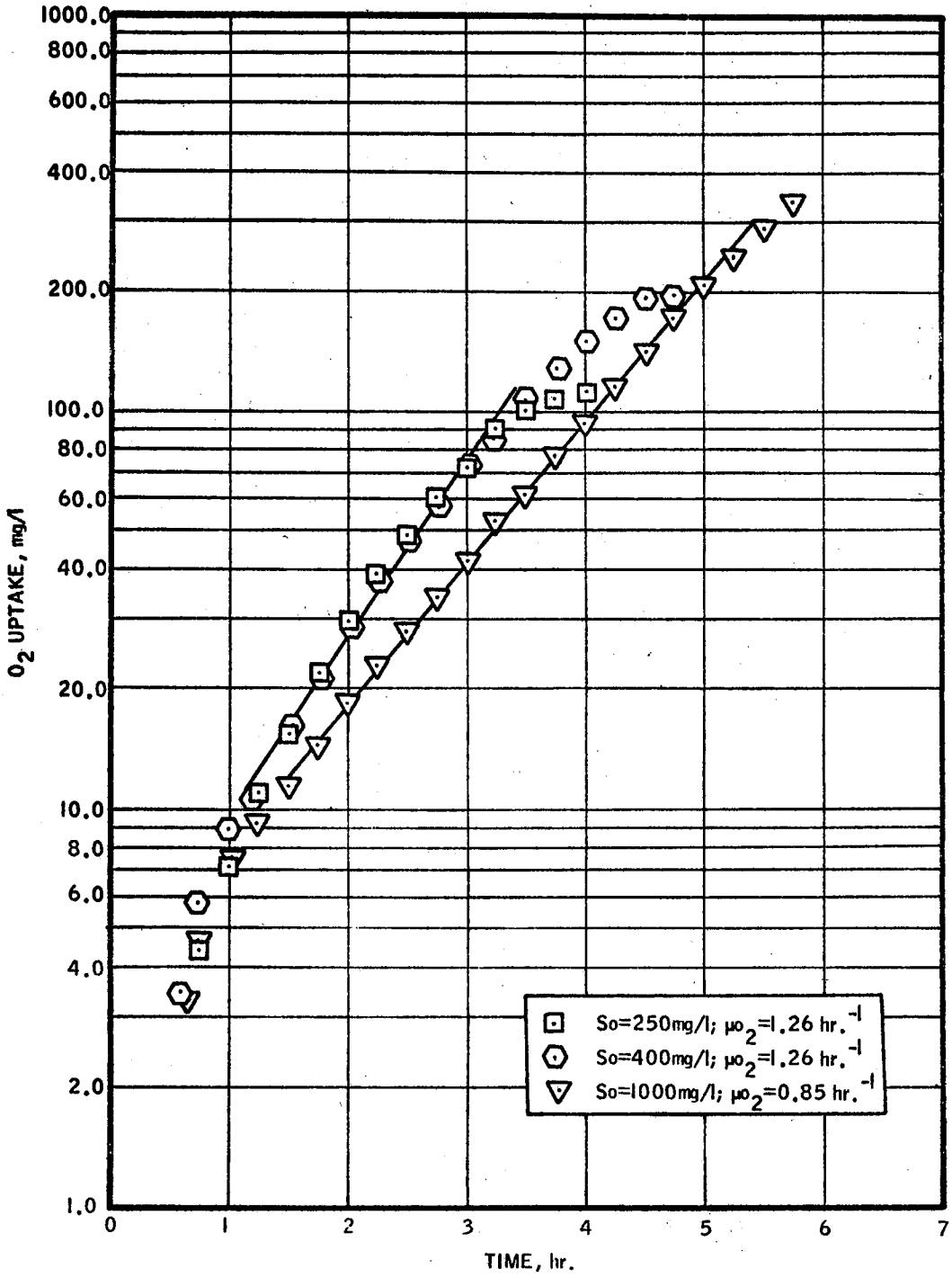



Figure 9. Logarithmic $O_2$ Uptake vs. Time for Experiment 10, With $S_o$ = 250 mg/l, and $S_o$ = 1000 mg/l.

by dilution (i.e., substrate concentration), but there were not enough data points at the lower substrate concentrations to make a definite statement in this regard or to define any possible relationships with $\mu_{O_2}$ and $S_o$. Because it is difficult to determine accurately low amounts of oxygen uptake on the Warburg apparatus, a microbial population exhibiting a high $K_s$ value would be an ideal system for study. Such a system was obtained by using a pure culture of Flavobacterium sp. The pure culture was obtained from the bioengineering laboratories at Oklahoma State University, and $K_s$ and $\mu_{max}$ were found to be 100 mg/l and .38 $hr^{-1}$, respectively, by Gaudy, Obayashi, and Gaudy (30). The oxygen uptake data for experiments 12 through 14 are shown on semi-logarithmic plots in Figures 10 through 12, respectively. The data from these experiments are tabulated in Table II.

As can be seen from Figures 10 through 12, all substrate concentrations exhibited fairly well defined logarithmic oxygen uptake phases. As observed in previous experiments, the oxygen uptake curve bent gradually into the logarithmic phase, i.e., the early portions of $O_2$ uptake exhibited ever decreasing rate of uptake until the relatively constant (exponential) rate was attained. These figures indicate a definite relation between oxygen uptake rates and dilution (substrate concentration). The composite results of these three experiments are presented in Figure 13, which shows plots of $\mu_{O_2}$ vs. $S_o$ and $1/\mu_{O_2}$ vs. $1/S_o$. The values obtained for $K_s'$ and $\mu_{O_2 max}$ from the straight line plot are 33 mg/l and 0.53 $hr^{-1}$, respectively. These results were substituted into equation (1), and the hyperbolic curve generated from this equation is shown in the plot of $\mu_{O_2}$ vs. $S_o$. From this plot it can be seen that equation (1) does provide a reasonably good representation of the

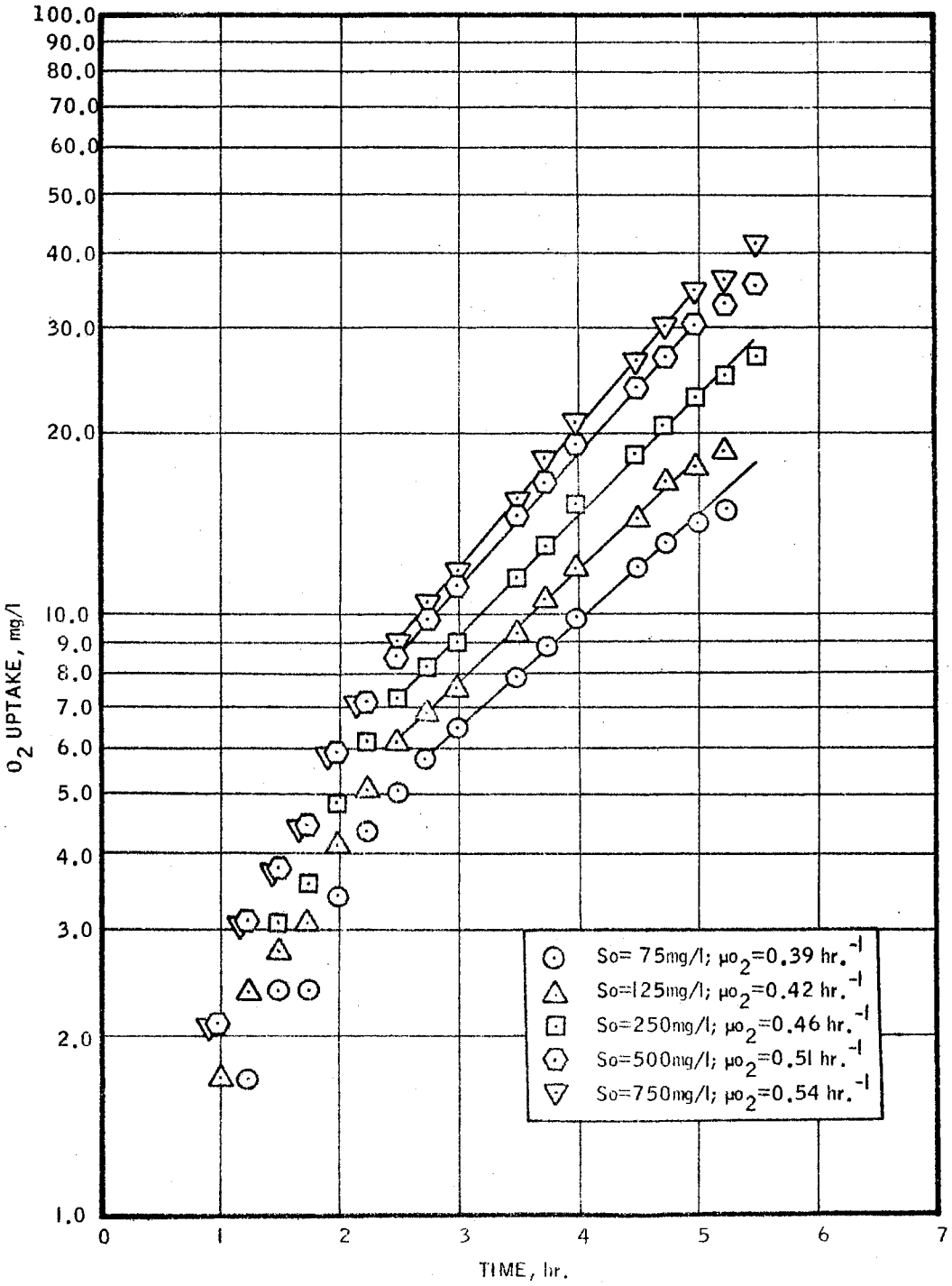



Figure 10. Logarithmic $O_2$ Uptake vs. Time for Experiment 12.

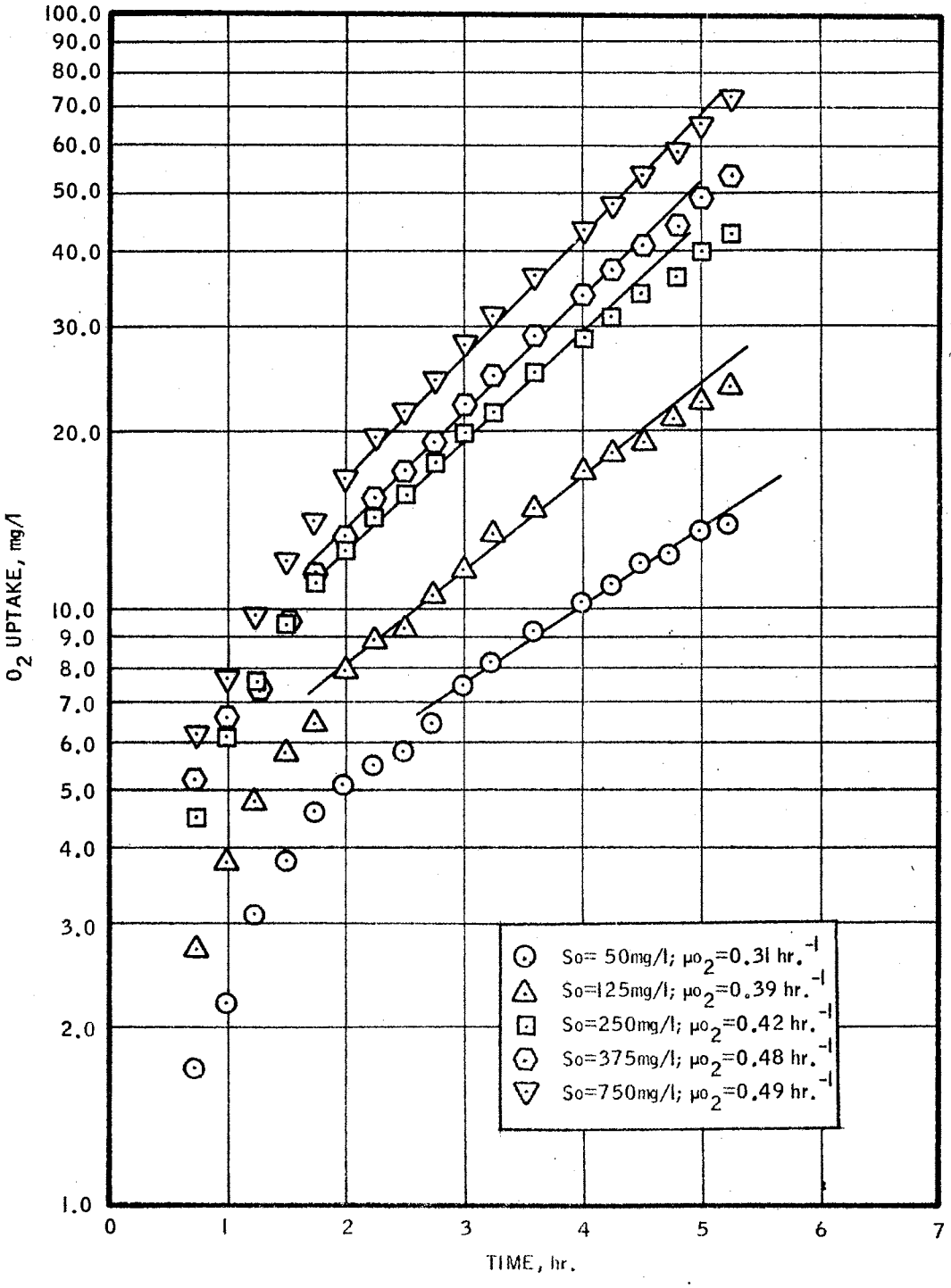



Figure 11. Logarithmic $O_2$ Uptake vs. Time for Experiment 13.

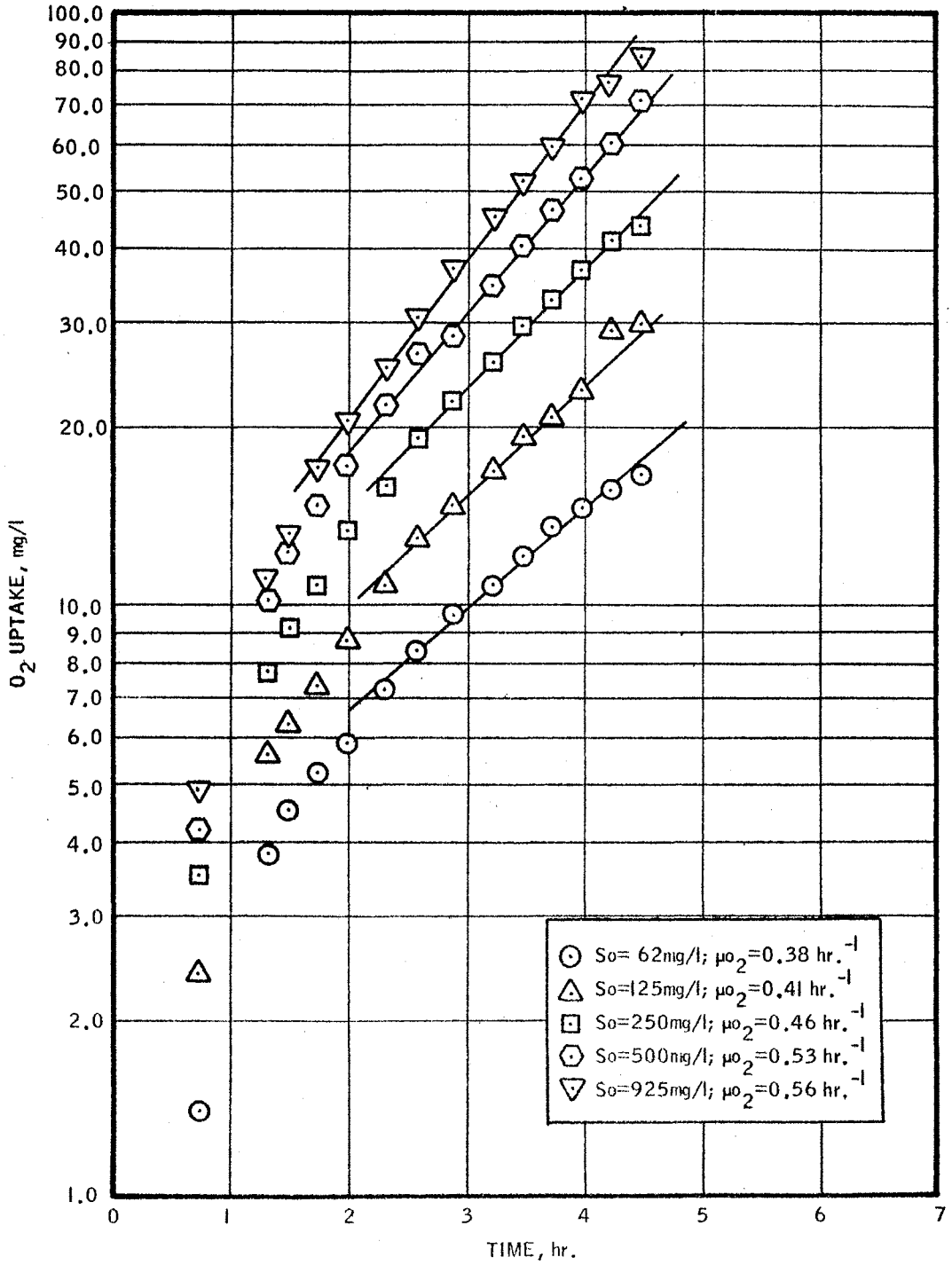



Figure 12. Logarithmic $O_2$ Uptake vs. Time for Experiment 14.

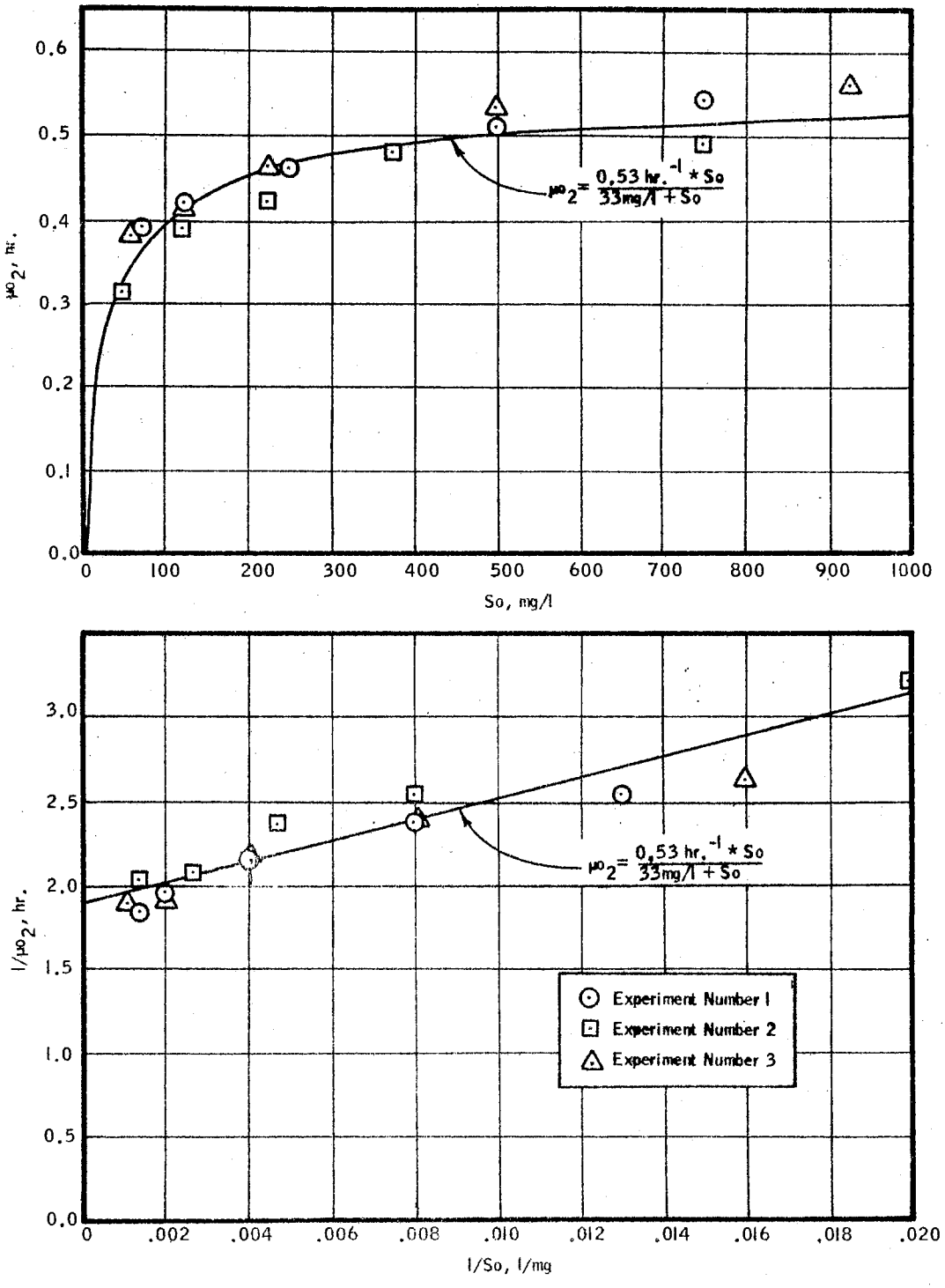



Figure 13. $\mu_{O_2}$ vs. $S_o$ and $1/\mu_{O_2}$ vs. $1/S_o$ for Experiments 12-14.

data. It is interesting to note that the dilution appears to affect $\mu_{O_2}$ less than it affects $\mu$; i.e., compare $K_s$ = 100 mg/l and $\mu_{max}$ = 0.38 $hr^{-1}$, and $K_s'$ = 33 mg/l and $\mu_{O_2max}$ = 0.53 $hr^{-1}$. This finding is consistent with the results presented earlier which indicated that $\mu_{O_2max}$ was attained at fairly low substrate concentrations.

IV. Experiment Relating Growth of Flavobacterium sp. to $O_2$ Uptake

Experiment 15 was run to aid in determining which portion of the oxygen uptake curve generated in the Warburg apparatus should be used to determine $\mu_{O_2}$ for Flavobacterium sp. Also, this experiment was run to gain insight into the possible causes for the constantly decreasing slope of the $O_2$ uptake curve (semi-logarithmic plot) as it approached the logarithmic phase of $O_2$ uptake. It is recognized that this phenomenon is the exact opposite of the lag phenomenon, a period of gradually increasing rate observed when measuring growth rather than $O_2$ uptake. Figures 14 and 15 show the results of this experiment, and the data obtained in this experiment are tabulated in Table III.

The results of the growth experiment and of the oxygen uptake experiment are presented in Figures 14 and 15, which show semi-logarithmic plots of optical density vs. time, and accumulated $O_2$ uptake vs. time, respectively. From Figure 14 it can be observed that growth experienced approximately a 2-hour lag phase before entering the logarithmic growth phase. At all substrate concentrations used, a definite logarithmic phase was established for growth. The growth experiment displayed a definite increase in $\mu$ with each increase in $S_o$, with $\mu$ ranging from 0.14 $hr^{-1}$ at 60 mg/l to 0.47 $hr^{-1}$ at 1000 mg/l. From Figure 15 it can be seen that while the growth curve exhibited a "lag," the oxygen uptake curve increased rapidly at a decreasing rate. However,

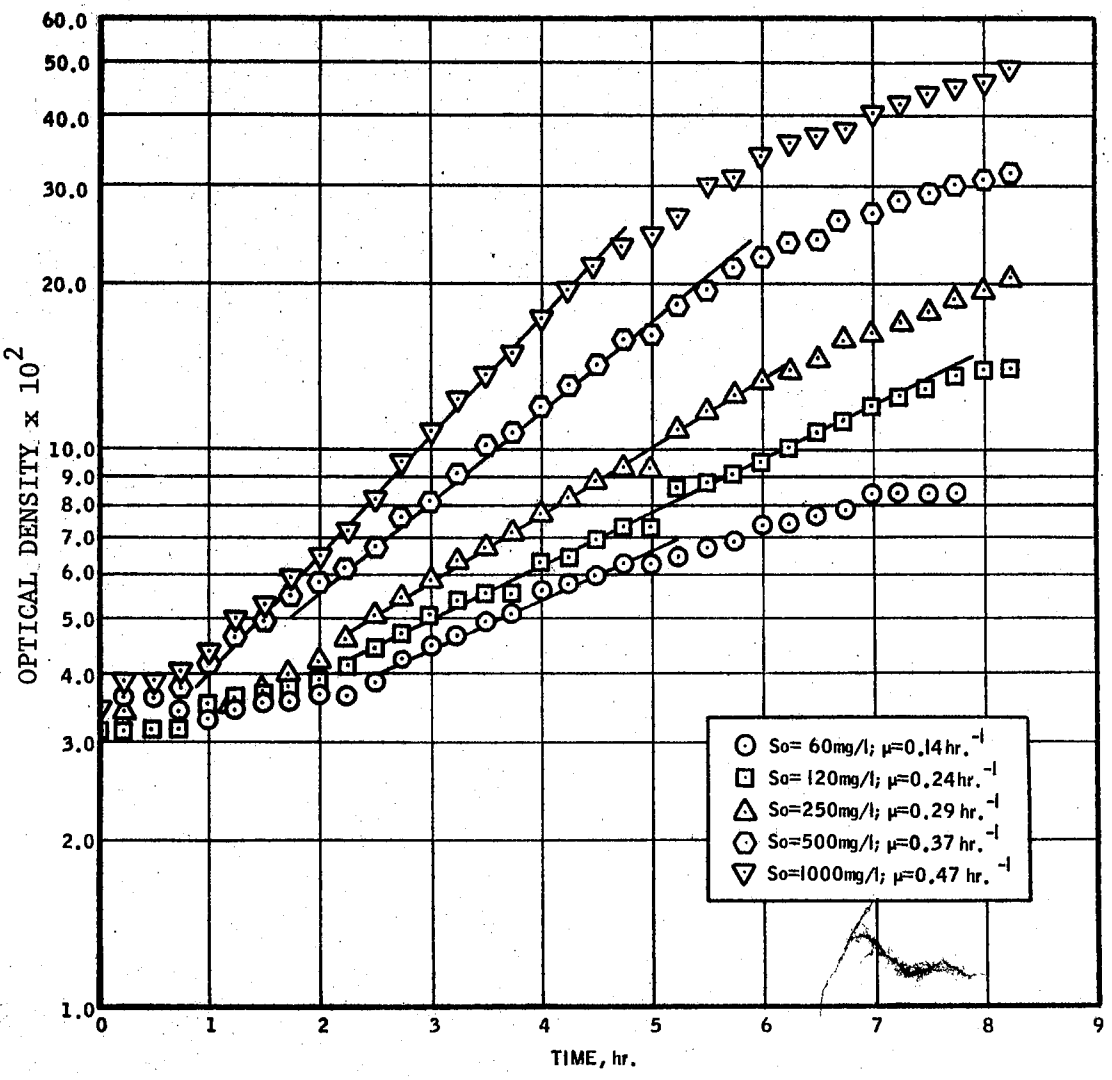



Figure 14. Logarithmic Optical Density vs. Time for Experiment 15.

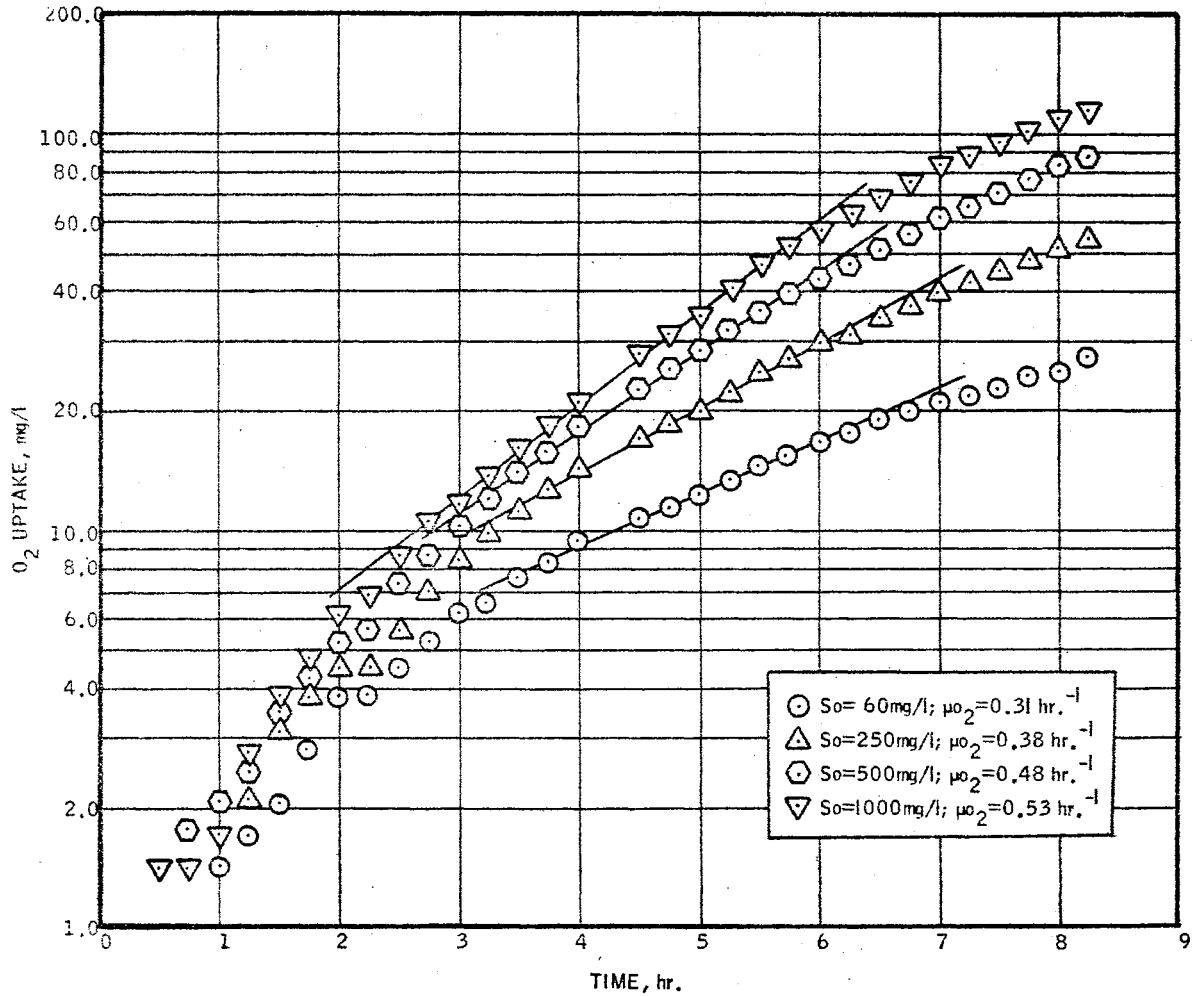



Figure 15. Logarithmic $O_2$ Uptake vs. Time for Experiment 15.

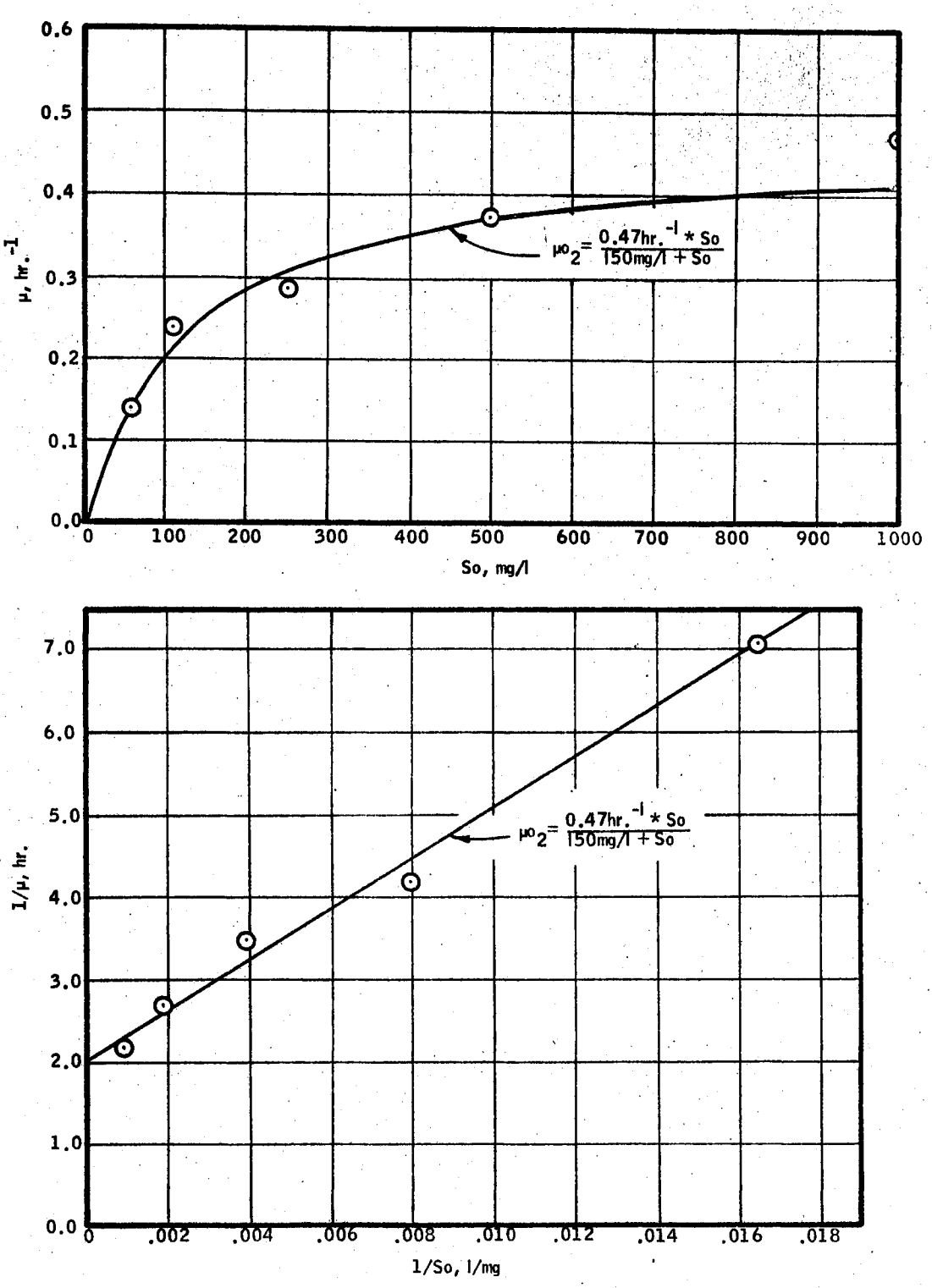
TABLE III

COMPARISON OF $\mu$ AND $\mu_{O_2}$ AT DIFFERENT VALUES OF $S_o$ FOR Flavobacterium sp. (Experiment 15)

| $S_o$ (mg/l) | $\mu$ ($hr^{-1}$) | $\mu_{O_2}$ ($hr^{-1}$) |
|---|---|---|
| 60 | 0.14 | 0.31 |
| 120 | 0.24 | * |
| 250 | 0.29 | 0.38 |
| 500 | 0.37 | 0.48 |
| 1000 | 0.47 | 0.53 |

*KOH spilled over into the reaction liquor and killed the organisms.

a definite logarithmic phase is observed in oxygen uptake, usually starting approximately one-half to one hour behind the start of the logarithmic phase for growth. Thus, it appears that in the lag phase of growth, $O_2$ uptake proceeds at a decreasing rate which eventually becomes exponential or, in any event, approaches a rate which can be characterized by the slope of a straight line on a semilogarithmic plot. The $\mu_{O_2}$ values for this experiment are consistent with those found in earlier experiments, with the range being from .31 $hr^{-1}$ at 60 mg/l to 0.53 $hr^{-1}$ at 1000 mg/l. $\mu_{O_2 max}$ and $K_s'$ for this organism were found to be 0.53 $hr^{-1}$ and 33 mg/l, respectively, in part 3(C) of this section (see above). Figure 16 shows plots of $\mu$ vs. $S_o$ and $1/\mu$ vs. $1/S_o$. The values obtained for $\mu$ and $K_s$ from the straight line plot are 0.47 $hr^{-1}$ and 150 mg/l, respectively. These results were substituted into equation (1), and the hyperbolic curve generated from this




Experiment 16. $\mu$ vs. $S_o$ and $1/\mu$ vs. $1/S_o$ For Experiment 15.

equation is shown in the plot of $\mu$ vs. $S_o$. From this plot it can be seen that equation (1) provides a reasonably good representation of the data. Thus, as suggested earlier, the change in substrate concentration did not affect $\mu_{O_2}$ as much as $\mu$, as evidenced by a $K_s'$ of 33 mg/l and $K_s$ of 150 mg/l.

V. BOD Bottle Experiments

Figures 17 and 18 show the results of experiments 17 and 23, respectively. The data from this set of experiments are tabulated in Table IV. In general, the results indicated an increase in $\mu_{O_2}$ with an increase in substrate.

Figure 17 shows results that are representative of experiments 16-21. In these experiments, enough seed was used in inoculating the BOD bottles to produce an optical density of approximately 0.045 on a Bausch-Lomb Spectrophotometer at a wavelength of 540 nm. The first portion of the curve could be a manifestation of the same type of decreasing rate phenomenon, as the curve approached the exponential range, which was observed in some of the experiments previously shown. Also, experimental difficulty was encountered in attempting to measure the DO during the first part of the experiments (when the DO was high).

In an attempt to gain more information with regard to the early rapid phase of $O_2$ uptake, two experiments (22 and 23) were run with a low seed (1.0 percent, or an optical density of 0.01). Experiment 22 was a preliminary experiment to determine if the high seed concentration could be the cause of the rapid initial oxygen uptake in the earlier experiments. One set of bottles was filled with the dilution water only; the second set with 5 percent seed (an optical density of approximately 0.045) and dilution water; and the final set with one

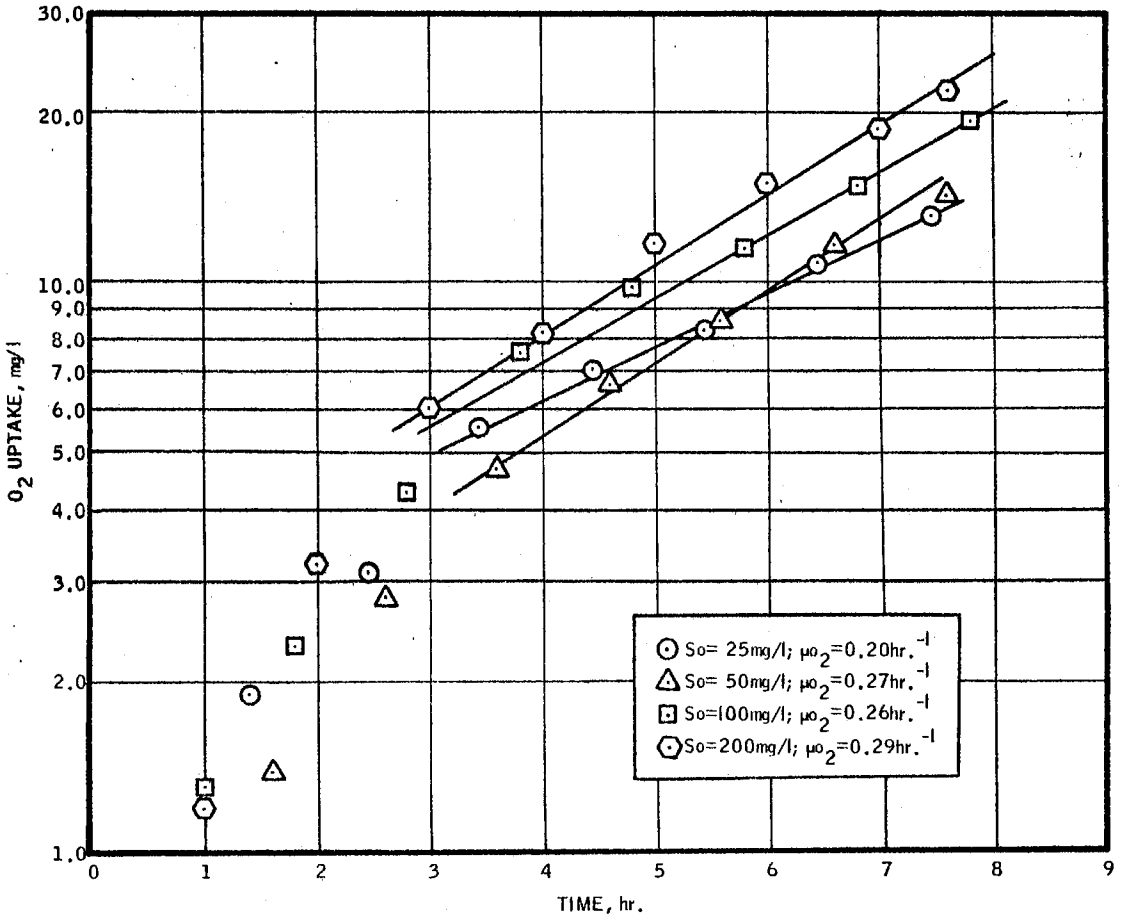



Figure 17. Logarithmic $O_2$ Uptake vs. Time for Experiment 17.

percent seed and 50 mg/l of substrate. During the time the DO was measured, the bottles with a 5 percent seed showed no oxygen uptake. However, the bottles with one percent seed and 50 mg/l substrate exhibited a logarithmic phase without the high initial oxygen uptake. This experiment provided some indication that the high initial seed could possibly be a cause for the two apparent logarithmic phases and/or the rapid initial oxygen uptake which gradually became exponential. Another experiment was therefore run, employing a low seed inoculum.

TABLE IV

RELATIONSHIP BETWEEN $\mu_{O_2}$ AND $S_o$ DURING EXERTION OF BOD

(Experiments 16-23)

| Exp. # | S (mg/l) | $\mu_{O_2}$ ($hr^{-1}$) | Exp. # | S (mg/l) | $\mu_{O_2}$ ($hr^{-1}$) |
|---|---|---|---|---|---|
| 16 | 50 | .2 | 20 | 16.5 | .22 |
| | 100 | .3 | | 33.0 | .47 |
| | 200 | .34 | | 50.0 | .46 |
| 17 | 25 | .2 | | 165 | .42 |
| | 50 | .27 | 21 | 16 | - |
| | 100 | .26 | | 40 | .235 |
| | 200 | .29 | | 60 | .32 |
| 18 | 40 | .14 | 22 | 50 | .09 |
| | 80 | .18 | 23 | 20 | .07 |
| | 400 | .22 | | 40 | .07 |
| 19 | 33 | .266 | | 80 | .094 |
| | 67 | .33 | | 160 | .154 |
| | 100 | .33 | | | |
| | 333 | .5 | | | |

Figure 18 shows the results of experiment 23, with the exception of $\mu_{O_2}$ at 20 mg/l, as it followed the same course as 40 mg/l. Experiment 23 was performed using a one percent initial seed, and as can be seen from this figure, the logarithmic phases were much more distinct than in the systems in which a high seed was employed. Only one logarithmic phase was evident. Also, the logarithmic phases exhibited are not as rapid as those exhibited in previous experiments in which higher initial seeds were used.

These experiments all exhibited logarithmic phases, and they generally indicated that $\mu_{O_2}$ would increase with an increase in substrate. However, it was not possible to establish a definite relationship, due partly to the experimental difficulty in the experiments using a high seed.

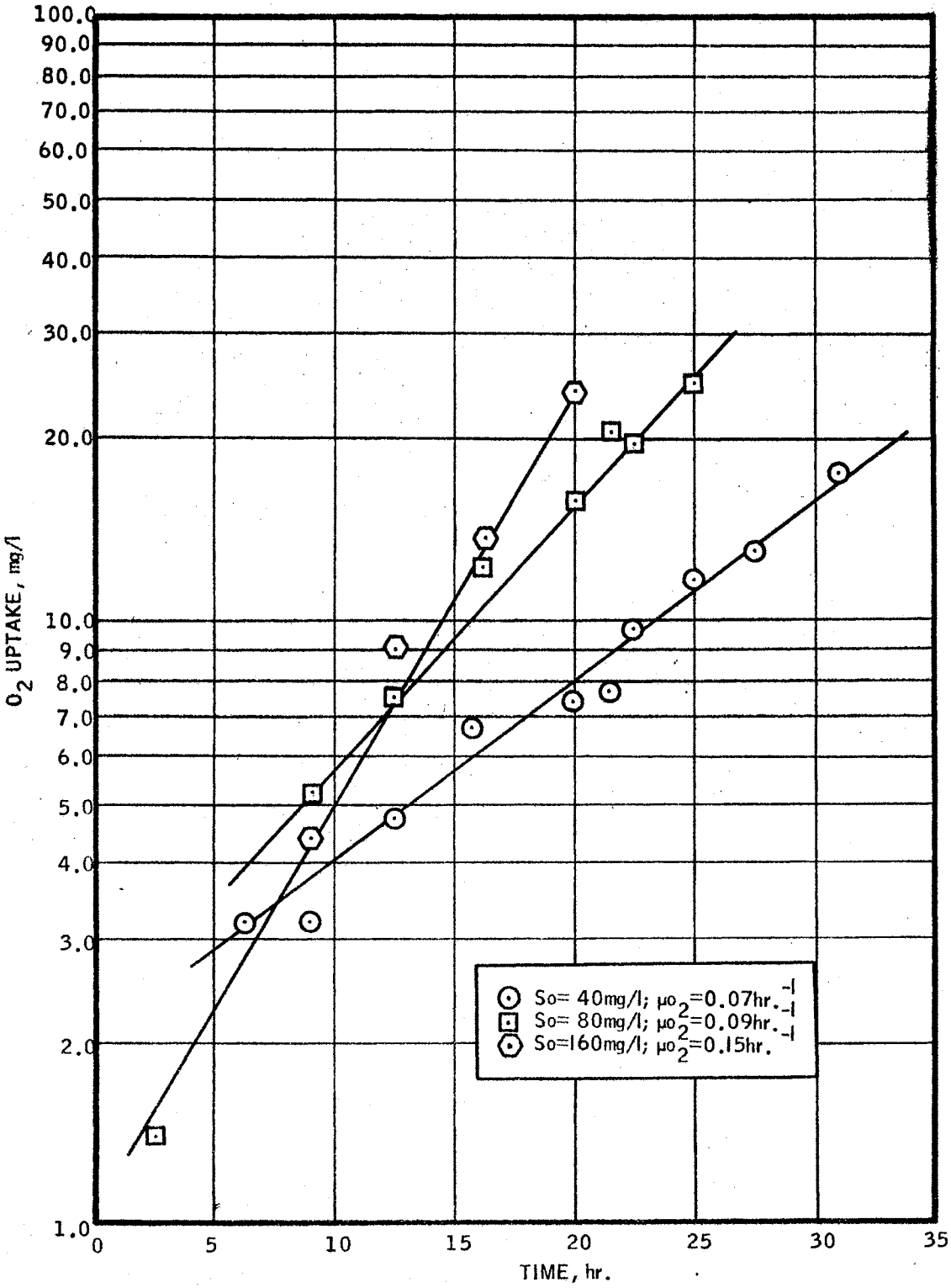



Figure 18. Logarithmic $O_2$ Uptake vs. Time for Experiment 23.

# CHAPTER V

## DISCUSSION

None of the experiments herein reported exhibited the "monomolecular" reaction kinetics which have been traditionally assumed to describe exertion of biochemical oxygen demand, i.e., $O_2$ uptake. In fact, as evidenced by the literature cited in Chapter II, many recent investigations have shown that monomolecular or first-order decreasing rate kinetics are not generally observable. In this investigation it was found that the oxygen uptake curve generally followed the pattern of the microbial growth curve except for a short early phase of uptake which gradually "tailed off" with exponential uptake. Although the logarithmic growth phase accounted for a relatively small portion (25-50 percent) of the total amount of oxygen ultimately used in metabolizing the synthetic waste, it is important to consider this rapid rate portion of the oxygen uptake curve in comparison to the overall oxygen uptake curve and its relation to analysis of the "DO sag curve" in receiving streams. Thus, due to the relatively rapid rate of oxygen uptake during the logarithmic phase of oxygen uptake, the end of the logarithmic phase lies close to the time required to attain the critical deficit, as shown by the battery jar experiments in this investigation. This observation is supported by findings of Gates, Marlar, and Westfield (31), and by Isaacs and Gaudy (32). Gates, et al. found

that in all instances, the rate of substrate utilization and the rate of oxygen uptake initially increased with decreasing substrate concentration until an inflection point was developed at some substrate concentration. The critical deficit never occurred before the inflection point in the substrate utilization curve. Isaacs and Gaudy (32) found that the critical deficit occurred at the beginning of the plateau region of the oxygen uptake curve which immediately follows the logarithmic phase. However, it should be pointed out that there are conditions under which oxygen uptake can proceed without a logarithmic curve. Two examples are $O_2$ uptake curves developed under severe nitrogen limitation and those representing endogenous respiration.

The standard dilution technique, which must be employed for most wastes, assumes that in the course of events the dilute system of the BOD bottle proceeds with the same kinetics as the course of events in natural systems to which the BOD test data are applied. On the contrary, this study indicates that dilution does affect the kinetics of the system, particularly in the range of dilute concentrations at which the BOD test is run. The data herein reported indicate that the "Monod equation" approximates the relationship between logarithmic oxygen uptake constant ($\mu_{O_2}$) and the initial substrate concentration ($S_o$). This finding is supported by Jennelle and Gaudy (25), who found that the "Monod equation" approximated their results when $\mu_{O_2}$ vs $S_o$ was plotted. The "Monod equation" with respect to $O_2$ uptake can be given as

$$\mu_{O_2} = \frac{\mu_{O_2 max} S_o}{K_s' + S_o}$$

The saturation constant, $K_s'$, was generally found to be rather small, indicating there would be large changes in $\mu_{O_2}$ with small changes in $S_o$

at low substrate concentrations. Thus, evidence has been presented indicating that the kinetic constants found using the BOD dilution technique cannot be expected to yield a usable approximation when the constants are inserted into any sag curve calculation, regardless of what the kinetics of the system are, unless the $S_o$ is the same for both systems.

The experiments using BOD bottles generally exhibited lower $\mu_{O_2}$ values than were found in the Warburg studies. In fact, there was a large difference in $\mu_{O_2}$ values between the experiments using high initial seed concentrations and the experiments using a low initial seed concentration. Since Flavobacterium sp. was used as seed in some Warburg experiments and all of the BOD bottle experiments, the comparison should be valid. It is suggested that two factors could cause the difference in $\mu_{O_2}$ values: (1) greater mixing in the Warburg, and (2) differences in initial seed concentrations. The $\mu_{O_2}$ values in the BOD systems using approximately the same initial seed concentrations as the Warburg experiments were close to the $\mu_{O_2}$ values in the Warburg system. The greatest difference between the $\mu_{O_2}$ values occurred with different amounts of initial inocula. This finding is reinforced with findings by Butterfield and Wattie (33), and by Jennelle and Gaudy (25). Butterfield and Wattie found that the initial bacteria concentration had a very marked effect on the amount of oxygen required during the early hours of the test. Jennelle and Gaudy (25) found that the degree of agitation or mixing employed in their experiments did not affect the oxygen uptake. They employed identical concentrations of the same seed population in both open and closed systems.

The experiments correlating oxygen uptake to growth illustrate

that the oxygen uptake curve does follow the growth curve, as mentioned earlier in Chapter II. However, the phase of oxygen uptake preceding the exponential phase was characterized by a curve with a constantly decreasing slope (when plotted on semilogarithmic paper). There appear to be two possible reasons for this effect: (1) the oxygen uptake starts at zero, and zero can never be plotted on semi-logarithmic paper, and (2) while the microbial population is undergoing a lag in synthesis and replication, a high respiratory requirement may be necessary in preparation for growth. After the lag phase in growth, the oxygen uptake curve closely followed the growth curve, with the exception that $\mu_{O_2}$ was less responsive to changes in $S_o$ than was $\mu$ in the range of substrate concentrations used.

The results of this investigation indicate that the present BOD dilution technique for use in studies on assimilation capacity of receiving waters is inadequate. The test should be modified so that it is more closely related to the natural system, particularly with respect to concentrations of substrate and type of seed. Such a system has been proposed by Gaudy (34) and is currently under investigation by Peil (35). In this system, an open stirred vessel is employed (similar to the one used in Chapter IV, section 2 of the current study.) The $K_2$ values (reaeration constants) are adjusted so that they are in the range of those expected in the receiving stream. One can employ as dilution water a sample directly from the receiving stream, and the sample can be run at the design temperature and dilution ratio expected in the stream. The DO profile for the system is then determined. From the known $K_2$ value and the DO profile, the oxygen uptake can be calculated and the oxygen uptake curve determined. In his

studies to date, Peil has obtained rather good correlations between the DO sags predicted using this method and those obtained in an artificial river in the bioenvironmental laboratories at Oklahoma State University.

# CHAPTER VI

## CONCLUSIONS

1. The monomolecular relationship does not approximate all oxygen uptake reactions. In the present study, a logarithmic relationship was observed in all cases.

2. The standard dilution technique is not suitable for the purposes for which the BOD concept is employed. Dilution did affect the oxygen uptake rate constant, and the relationship follows the hyperbolic form:

$$\mu_{O_2} = \frac{\mu_{O_2 max} S_o}{K_s' + S_o}$$

3. Although the oxygen uptake rate was affected by dilution (i.e., $S_o$), the effect was not nearly so great as the effect of $S_o$ on microbial growth rate.

4. In the experiments using different initial concentrations of Flavobacterium sp., the experiments with a higher initial seed exhibited higher $\mu_{O_2}$ values.

# CHAPTER VII

## SUGGESTIONS FOR FUTURE WORK

The following future investigations are suggested:

1. More work should be done correlating microbial growth and oxygen uptake to help elucidate the cause of the differing kinetics in "growth" and $O_2$ uptake in the phase preceding exponential growth.

2. Further work correlating microbial growth and oxygen uptake might delineate quantitative relationships which could allow one to predict or estimate growth by determining $O_2$ uptake, or vice versa. Such relationships might also aid in predicting the sludge yield.

3. More work should be done to determine how the initial seed affects the $\mu_{O_2}$ values at various levels of $S_o$.

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