STUDIES ON NATURAL MICROBIAL POPULATIONS I. DETERMINATION OF μ_{max} AND k_s FOR VARIOUS SUBSTRATES, INCLUDING MUNICIPAL SEWAGE II. THE EFFECT OF SODIUM AND CHLORIDE IONS ON GROWTH, SUBSTRATE REMOVAL, AND CELL YIELD

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PART I

DETERMINATION OF μ_{max} AND k_s FOR VARIOUS SUBSTRATES, INCLUDING MUNICIPAL SEWAGE

CHAPTER I

INTRODUCTION

The trend in designing biological waste water treatment plants is toward the use of completely mixed reactors. This type of reactor allows the process to approach a steady state condition with respect to carbon source and biological solids concentration. Microbiologists, working with pure cultures, have accomplished much research on biological reaction kinetics, while workers in the pollution control field are just now learning that a steady state can be approached with heterogeneous populations.

From previous work accomplished in this bioengineering laboratory it has been concluded that a steady state condition (with respect to cell and substrate concentrations) can be approached for completely mixed reactors in systems where sludge recycle is not practiced (1). Also it has been found that a steady state can be approached in systems employing cell recycle (2).

Several parameters are included in kinetic equations for microbial growth in a steady state condition, e.g.,

growth rate, substrate concentration, and saturation constant. In the pollution control field two relationships are considered which describe the relation between growth rate and substrate concentration. The first is a single phase concept embodied in the Monod equation (3) and the second involves a two phase linear relationship described by Garrett and Sawyer (4). A considerable amount of work has been accomplished in both the microbiology and pollution control fields on one or the other of these concepts. All work done thus far in this laboratory has resulted in support of the Monod relationship. Quoting from an article by Gaudy and Gaudy (5), "While there is increasing need for mathematical formulations of predictive value, there is an even greater need for accumulation of sufficient data, obtained under controlled conditions, to test the validity of models, equations, and theories which are often proposed and accepted with a minimum of experimental verification." The present study was designed to obtain data on the growth of heterogeneous populations for several substrates and to determine the applicability of either kinetic concept to heterogeneous microbial systems.

The present federal law is such that all organic waste will require secondary treatment before waste water may be discharged into a stream. As the population increases a higher degree of treatment may be required. In order to improve the reliability of secondary treatment

(biological) it would surely seem that traditional design and operational criteria need to be revised. Such revisions cannot come about without laboratory and field research.

CHAPTER II

LITERATURE REVIEW

The present trend away from the standard plug flow aeration tank to the completely mixed tank has come about in part because of the development of steady state equations describing continuous cultures of microorganisms. Theoretically, in a completely mixed reactor at steady state the rate of change in cell concentration and substrate concentration is zero. The steady state can be closely approached for pure cultures (6). Based upon their experimental results Gaudy, Ramanathan, and Rao (1), having concluded that for a completely mixed once-through type reactor, a steady state with respect to the biomass (heterogeneous populations) could be approached, not attained. In later work Ramanathan and Gaudy (2), using systems employing cell feedback, have shown the existence of a pseudo steady state condition and have proposed modifications to Herbert's (6) equations.

In order to solve steady state equations employing the single phase model relating logarithmic growth rate, μ, two "constants" which must be determined are maximum

growth rate, μ_m (hr^{-1}), and saturation constant, k_s (mg/l) since these "constants" are used in describing the relation between growth rate, μ, and substrate concentration, S. According to Monod this relation may be described by the following equation (3): $\mu = \mu_m S/k_s+S$.

Garrett and Sawyer concluded that the following relationship existed: $\mu = KS$. In the relationship μ increases linearly with S up to a certain substrate concentration at which μ no longer increases, i.e., μ_m is finally attained and further increases in S have no effect on the growth rate constant. They plotted soluble BOD remaining versus dilution rate for experimental runs in continuous flow units operating at three detention times (i.e., dilution rates). From the three points a straight line was constructed and they concluded that a relation between growth rate and remaining soluble BOD could be described by a linear function. They proposed this discontinuous function for practical design purposes, but it was taken as kinetic theory by other workers. McCabe and Eckenfelder (7) (8) modified this concept, somewhat, to formulate design procedures for waste treatment. McKinney (9) has also used this relationship in equations for completely mixed actvated sludge units.

The Monod relationship (10) was proposed in 1942 and is now accepted by many microbiologists. In 1942 Monod was examining bacterial growth kinetics when he found that a plot of the logarithmic growth rate constant versus

initial substrate concentration yielded a rectangular hyperbola. He compared his curve with that of Michaelis-Menton, since this equation is of the same form as the one he obtained and he also compared their theory on enzymatic reaction rates with his ideas on bacterial growth rates. He did not make a direct correlation between the two equations and felt that his equation was an empirical one; it was later taken as theory by other workers.

Monod later stated, "There is little doubt that, as further advances are made towards a more integrated picture of cell physiology, the determination of growth constants should and will have a much greater place in the experimental arsenal of microbiology." (3) This prediction has indeed been realized in the field of microbiology, and is recently coming into focus in the pollution control field through expression of the various steady state completely mixed design equations which admit to the fact that the biomass is in a logarithmic growth phase.

In the microbiology field many authors accept the Monod relationship, but most have a slight modification for the equation. Painter and Marr (11) have pointed out the formulations for the mathematics of growth and division of the individual microorganisms which ultimately determines the behavior of the population. James (12) reviewed the work in the area of growth kinetics and said the data on the growth of a variety of microorganisms seem to fit Monod's equation for overall population

growth rather well. He also felt that the cell was an integrated entity and that the equation should account for the growth and division of the individual cell. Concerning the existence of a steady state within the individual cell he pointed out that these units are not invariant in their composition during division and, therefore, the condition cannot be precisely described as one of the steady state. Meers and Tempest (13) accepted the Monod equation but felt that it was inadequate for cultures in which growth is affected by bacterial products which accumulate in the environment. They recommended that a population-product factor should be added to the equation.

Northam (14) derived differential equations to describe the course of bacterial growth from inoculation to the achievement of a steady state level in a completely mixed continuous flow reactor. Applying his equations to the data of Herbert he was able to show (using the Monod model constants), in cases where the saturation constant, k_s, was small, that in steady state bacterial populations (a pure culture) the approximations to exponential growth hold for at least 99 per cent of the population. Thus his work tends to substantiate the applicability of the Monod equation for cultures at the steady state level. Other workers (15) (16) (17) have employed the Monod constant in assessing mathematically various models of kinetic hypotheses. It is significant, however, to find many authors working on kinetic formulations but very few reporting values for the

factors employed in the equations. The two factors of interest for this study were the maximum growth rate and saturation constant which are essential to use the Monod relationship. In the remaining portion of this part of the literature review some of the values found for these constants are given.

Herbert, Elsworth, and Telling (6) used a pure culture of Aerobacter clocae growing on 0.0272M glycerol to test the validity of Monod's concept. They reported mean values of μ_m = 0.85 (hr^{-1}) and k_s = 12.3 mg/l and concluded that growth could be described using Monod's concept. Also noted in their article were the values that Monod determined (10). These were k_s values for glucose = 4 mg/l, mannitol = 2 mg/l, and lactose = 20 mg/l. Monod reported that these values should be related to the apparent dissociation constant of the enzyme invc lved in the first step of the breakdown of a given compound. Also he felt that a change of conditions affecting the velocity of only one rate-determining step will be only partially reflected in the over-all rate, therefore, one might expect the k_s values to be lower than the corresponding values of the Michaeli's constant of the enzyme catalysing the reaction. He said this may explain why the values are so often so small compared to the concentrations required for visible growth.

Others reporting values for μ_m and k_s are Tempest and Hunter (18). Using glycerol they found $\mu_m = 0.45$ hr^{-1}. Wase and Hough (19) growing yeast on 0.01% phenol as sole carbon source found $\mu_m = 0.1133$ hr^{-1} and an average $k_s = 24.96$ mg/l. Dean and Rogers (20) used a pure culture of Aerobacter aerogenes grown in a magnesium limited system with 270 mg/l glucose as the carbon source and reported values for $\mu_m = 1.20$ hr^{-1} and $k_s = 23$ μM. Beck and von Meyenburg (21) used a pure culture of Saccharomyces cerevisiae grown on glucose and found $\mu_m = 0.42$ hr^{-1}. They also determined a value for $\mu_m = 0.14$ hr^{-1} using ethanol. Button and Garver (22) used a pure culture of Terulopis utilis to study the kinetics of oxygen limited growth. They used glycerol as a carbon source and found $\mu_m = 0.506$ hr^{-1} and $k_s = 49$ μM.

The above citations indicate that a considerable amount of work is being done pertaining to definitions of μ_m and k_s with pure cultures. The growth of one particular species organism on one substrate will not likely be the same as another organism growing on the same substrate. In the pollution control field values for these factors need to be determined for heterogeneous populations. It is only through such experimentation that the pollution control field can ever know if the kinetic concepts employed to describe microbial growth in selected systems can be employed in natural systems.

Gaudy, Ramanathan, and Rao (1) studied the kinetic behavior of heterogeneous populations in completely mixed reactors. They concluded that the single phase relationship of Monod was applicable to mixed populations. They used batch studies to determine the growth factors. The average values obtained with a readily metabolized carbohydrate (glucose) were $\mu_m = 0.53\ hr^{-1}$ and $k_s = 90$ mg/l.

In a more recent study by Ramanathan and Gaudy (2) the average values obtained on glucose were $\mu_m = 0.52\ hr^{-1}$ and $k_s = 57$ mg/l. In the same study they determined the constants for systems that employed cell recycle. The average values for glucose were $\mu_m = 0.47\ hr^{-1}$ and $k_s = 67$ mg/l. It was concluded from the previous two articles from the Oklahoma State University laboratories that for a readily available carbohydrate (e.g., glucose), a usable range of values for k_s, 75-125 mg/l, and μ_m, $0.5\text{-}0.6\ hr^{-1}$, can be considered as a guide for industrial wastes consisting primarily of carbohydrates. Because biological waste treatment involves heterogeneous populations that vary from day to day and from substrate to substrate, a usable range rather than precise numerical values should be given.

From the past work in this laboratory all the results have indicated the applicability of the Monod relationship for heterogeneous populations. It is now of impor-

tance to determine the range of values for μ_m and k_s for a variety of compounds as well as complicated carbon sources, e.g., municipal sewage.

CHAPTER III

MATERIALS ANI METHODS

A. Experimental Protocol

Natural microbial populations were grown up (in batch culture) on each carbon source from initial sewage seeds obtained from the municipal treatment plant at Stillwater, Oklahoma. All experiments were conducted at room temperature (23 $\pm$ 2°C) on a shaker operating at a rate of 90 oscillations per minute. All flasks contained 50 ml of reaction liquor (250 Erlenmeyerflasks). The composition of the synthetic waste and various carbon sources used are shown in Table I.

The acclimation procedure was as follows. Five ml of sewage seed were added to 45 ml of synthetic waste and this reaction liquor was aerated (shaken). Daily 5 ml of the cell suspension were transferred to a new flask containing 45 ml of fresh media. The cells were then acclimated to the substrate for a week prior to using them for an experiment. This stock system was maintained for several more weeks and then the cells were used for a second growth rate experiment. In a growth rate experi-

TABLE I

COMPOSITION OF GROWTH MEDIUM FOR 1000 mg/l SUBSTRATE AS GROWTH-LIMITING NUTRIENT

Constituents	Concentration	
Carbon Source[1]	1000	mg/l
Ammonium Sulfate, $(NH_4)_2SO_4$	500	mg/l
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	100	mg/l
Ferric chloride, $FeCl_3 \cdot 6H_2O$	0.50	mg/l
Manganous sulfate, $MnSO_4 \cdot H_2O$	10.0	mg/l
Calcium chloride, $CaCl_2$	7.5	mg/l
1.0 M Potassium Phosphate buffer ph 7.0 (KH_2PO_4 - 52.7 g/l) (K_2HPO_4 - 107 g/l)	10.0	ml/l
Tap water (Trace Elements)	100.0	ml/l
Distilled water	to volume	

[1]Glucose, Lactose, Sucrose, Sorbitol, Alanine, Glutamic acid, Serine, Histidine, Phenylalanine, Cysteine, Acetic acid, Propionic acid, and Sewage.

ment cells for the stock flask were inoculated into flasks containing fresh growth media at various concentrations of carbon source. The seeding population was not washed because it was known from a study of the growth curves that all substrate was removed in less than 24 hours and there was no danger of carrying over substrate to the experimental flask.

For the first few experiments nine concentrations of carbon source covering the range of 50 to 1000 mg/l were employed. Later the range was increased from 10 to 1500 mg/l and eleven flasks per experiment were used. Each flask was inoculated with 2.5 ml of seed culture from the stock system and the balance was made up to 50 ml with growth medium. The initial per cent transmittance was approximately 90%. The pH in all systems was approximately seven. The cysteine and propionic acid substrates were the only substrates which required an additional amount of buffer to raise the pH. The final set of experiments was accomplished using municipal sewage as the substrate. This sewage, collected from the effluent of the primary clarifier, was concentrated in order to obtain a COD high enough to cover a sufficient range of substrate concentration. The pH of the concentrated sewage was 8.5 and remained close to this in all units during the growth experiments. Since rapid and copius growth was obtained, the pH was not adjusted to seven. A sewage seed was acclimated to the concentrated sewage substrate a few days

before an experiment was run, and a new growth was started for the second experiment using another batch of concentrated sewage.

B. Concentration of Raw Sewage

1. Flash Evaporation

Several liters of effluent from the primary clarifier were collected and the COD was measured for both filtered and unfiltered samples. Five hundred ml portions of the sewage were evaporated (Buchler Instruments evaporator) under a reduced pressure at temperatures of 65°C and 55°C. The volume of sewage was reduced to one twentieth of the original volume. The stock concentrate was then centrifuged and filtered through a membrane filter. A COD measurement was taken before and after filtering. The resulting sewage substrate was yellow, and it was noted in preliminary experiments that the color did not change during the course of microbial growth. The filtrate was refrigerated at approximately 0°C until used in experimentation.

2. Freeze Drying

The techniques for lyphilization were discussed with several microbiologists and a laboratory unit was constructed. The procedure involves freezing a thin layer of sewage on the walls of a vacuum flask immersed in a bath containing a dry ice-acetone mixture. Under a vacuum the frozen water should then sublime off leaving

the dried or nearly dry sewage behind. The sublimed water vapor is condensed in a dry ice-acetone trap.

Several portions of sewage (5 ml in a 1000 ml flask) were frozen in a thin layer and then attached to the vacuum source. Each time, melting occurred before sublimation was noticed. With the proper equipment and vacuum source, the water should sublime immediately when subjected to the reduced pressure. Failure of the procedure was thought to be due to improper tubing in the set up (diameter too small) and not enough vacuum capacity. More is said concerning this technique in the discussion chapter.

C. Analyses

1. Biological Solids (Optical Density)

The optical density of the mixed liquor was employed to determine the course of growth in each system. Per cent transmittance was measured and converted to optical density using a chart, which had been prepared in accordance with the equation $OD = -\log_{10}T$. All measurements were made at a wave length of 540 nm using a distilled water blank. A Bausch and Lomb spectrophotometer was used.

The values for optical density were plotted on semi log paper (base 10) versus sample time for each concentration of initial COD. The slope of the straight line portion of the resulting curves gave the values for each

growth rate, μ, hr^{-1} (appropriately corrected to base e). This was done using the following equations; $\mu = (\ln X_1 - \ln X_2)/\Delta t$, where $\ln X_1 = 2(\ln X_2)$. The value for X_1 was chosen at twice the value for X_2. In this case $\mu = 0.693/\Delta t = hrs^{-1}$. A Lineweaver, Burk (23) plot and a Monod (3) plot were then made for each substrate from which the values for maximum growth rate, μ_m and the saturation constant, k_s were calculated.

The Lineweaver, Burk plot used is described by the equations; $1/\mu = 1/\mu_m + k_s/\mu_m(S)$, multiplying both sides of the equation by S yields the following relationship, $S/\mu = S/\mu_m + k_s/\mu_m$. This equation plots a straight line with S/μ on the ordinate and S on the abscissa. The slope of the line is $1/\mu_m$ and the intercept is k_s/μ_m. This type of plot was employed because it tends to spread out the points, and allows more accurate estimation of the slope than does the usual reciprocal plot of Lineweaver, Burk.

The direct plot of μ vs S (Monod plot) yields a hyperbolic curve in accordance with the equation: $\mu = \mu_m S/k_s + S$. The values for μ (ordinate) were plotted versus the values for S (abscissa). From this plot the values for μ_m and k_s can also be estimated (but not too accurately); it was used to determine if a single phase relationship for μ and S existed.

CHAPTER IV

RESULTS

Thirteen substrates were investigated. With one exception, two sets of growth experiments were run for all substrates. For comparative purposes all experiments on a particular substrate are shown on the same graph. All values for μ_m and k_s are listed in Table II at the end of the results section.

A. Carbohydrates

In the first four experiments the lowest substrate concentration used was 50 mg/l, and it was difficult to estimate k_s values since they appeared to lie near or below 50 mg/l. In order to express these low values, the remaining experiments included substrate concentrations of 10 and/or 20 mg/l.

1. Glucose

The second experiment using glucose (see #2 on figure 1) was accomplished three weeks after experiment one. During the time between experiments the color of the cells had changed from milky to green, indicative of a change in microbial predominance. The difference in

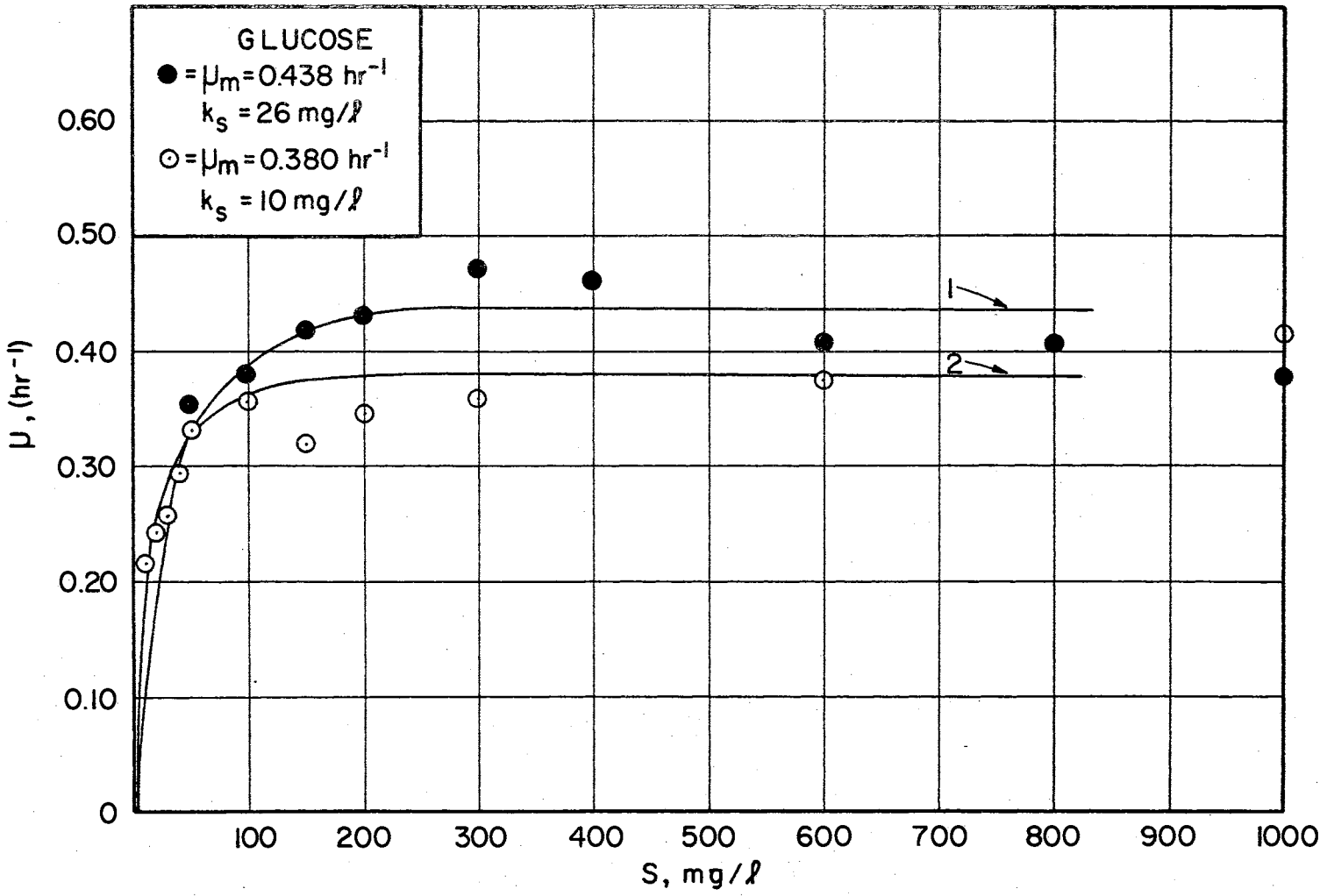

Figure 1. Monod Plots for Glucose

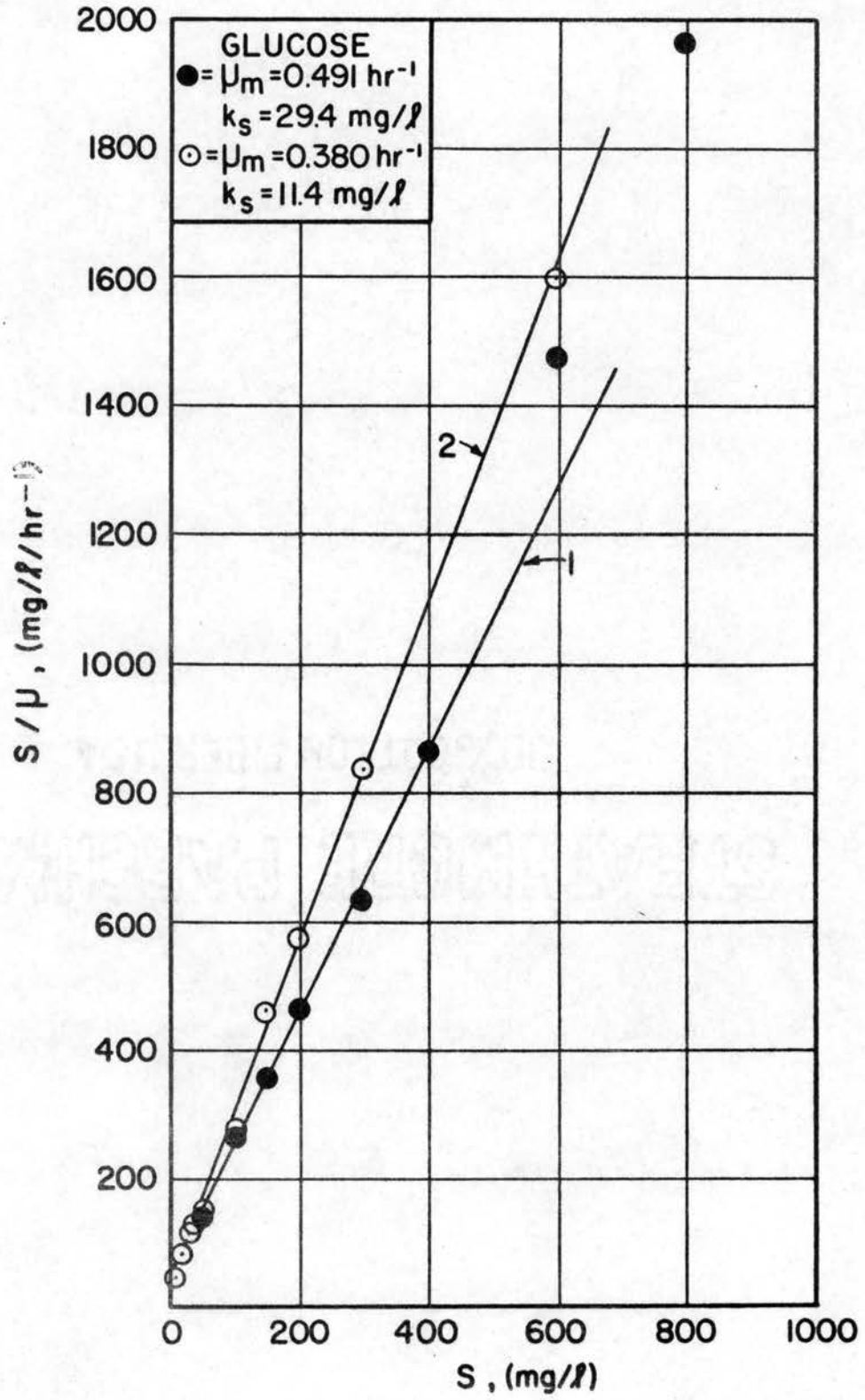

Figure 2. Lineweaver, Burk Plots for Glucose

maximum growth rate, for experiment 1 and 2 may have been due to a change in predominance. Figure 2 shows the Lineweaver, Burk plot for the same experiments on glucose. The tailing off of the values for the higher concentrations in the first run also caused these values to fall off the straight line plot. The values of μ_m and k_s calculated from this plot for the second experiment are very close to the values obtained from the Monod method of plotting.

2. Lactose

The four experiments on lactose are shown on figures 3 and 4. For the first experiments a 1½ hour lag was noted for the higher substrate concentrations and a 4 hour lag was observed in the flask containing 50 mg/l. During the second run there was a 2 hour lag phase. Between the first two runs, the turbid milky color of the bacterial suspension in the stock flask changed and the suspension could be described as a flaky or flocculated type of growth. The cells were resuspended in a blender before the second run. A third run was made with cells from the stock flask several weeks after experiment 2 and this time the 10, 20, 30, and 40 mg/l concentrations produced no growth in six hours. A Monod type curve could not be drawn through the points but a two phase linear plot was also improbable. It is felt that the data for this run should be disregarded in assessing the value or range of the kinetic constants. A new stock population was started from a sewage seed and after several days of acclimation

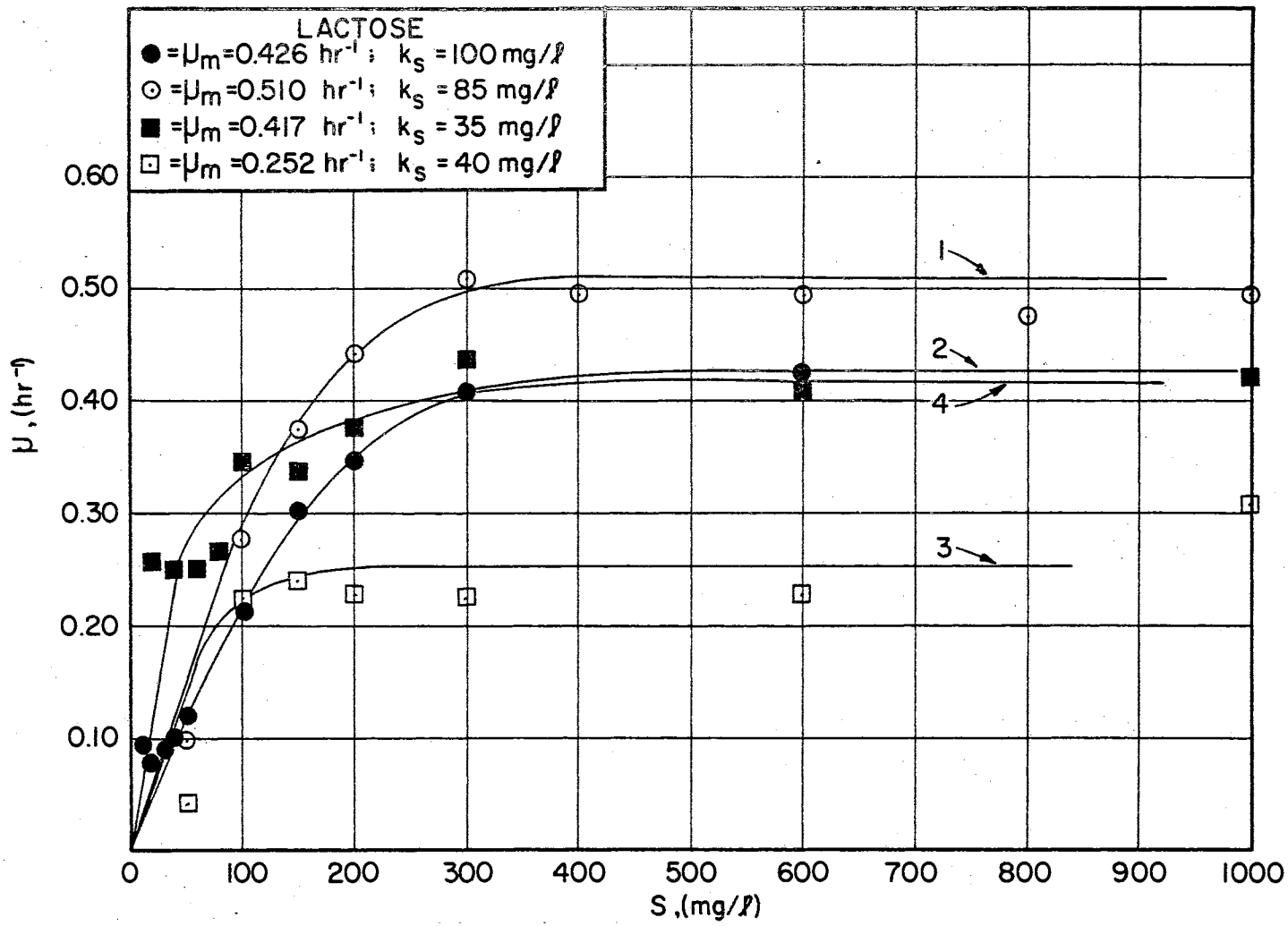

Figure 3. Monod Plots for Lactose

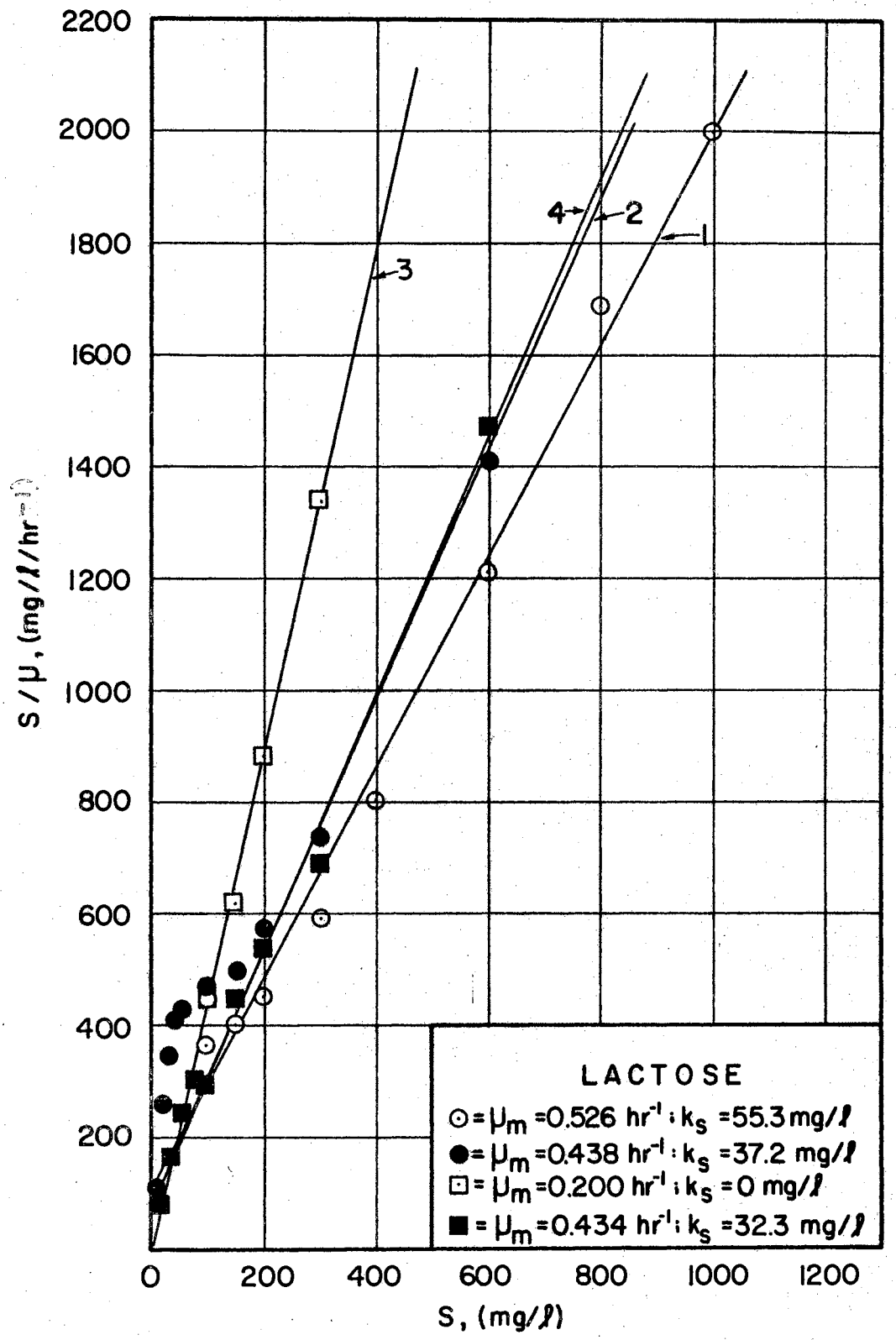

Figure 4. Lineweaver, Burk Plots for Lactose

to lactose experiment 4 was performed. The cell suspension exhibited a turbid milky appearance similar to the appearance of the suspension at the time of running experiment 1.

3. Sucrose

The results of the two growth experiments using sucrose substrate are shown in figures 5 and 6. Between the time of running experiments 1 and 2 there was a rather drastic change in microbial predominance as indicated by changes in the appearance of the mixed liquor in the stock population. At the time of the first sucrose run the suspension exhibited a milky appearance while at the time of the second run the mixed liquor had a greenish hue. Results of the first run indicated that lower substrate concentrations should be run. Such concentrations were included in the second run. The most striking difference between the two sets of results is the values obtained for μ_m.

4. Sorbitol

Throughout the period of experimentation using sorbitol there were no evidences of a gross change in predominant species, as indicated by the appearance of the mixed liquor; it retained a dispersed milky appearance. In figure 7 it is seen that the first run could have benefited if lower substrate concentrations had been employed. In the second run lower concentrations were included and the curve is rather well defined. A slight drop-off

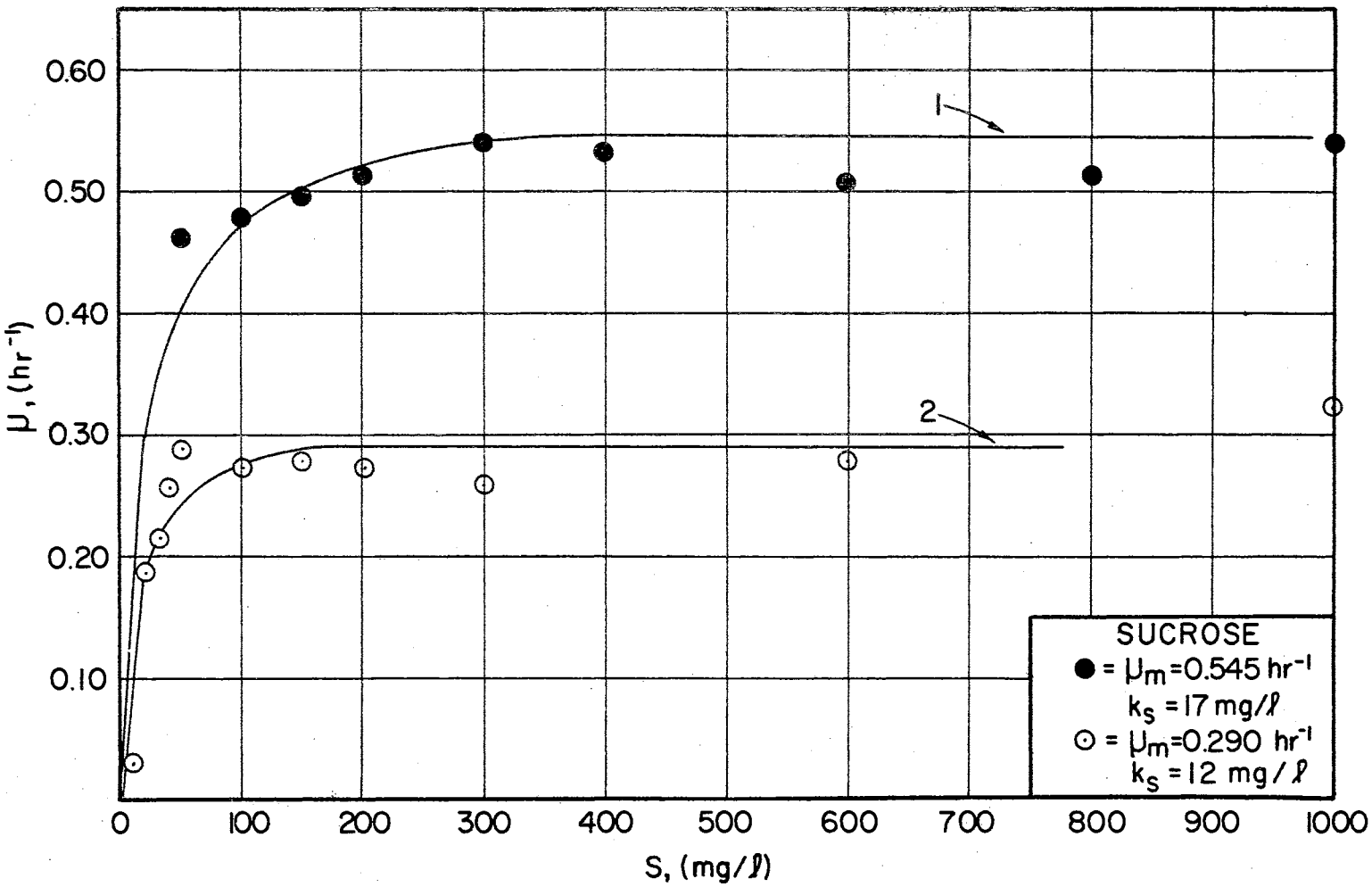

Figure 5. Monod Plots for Sucrose

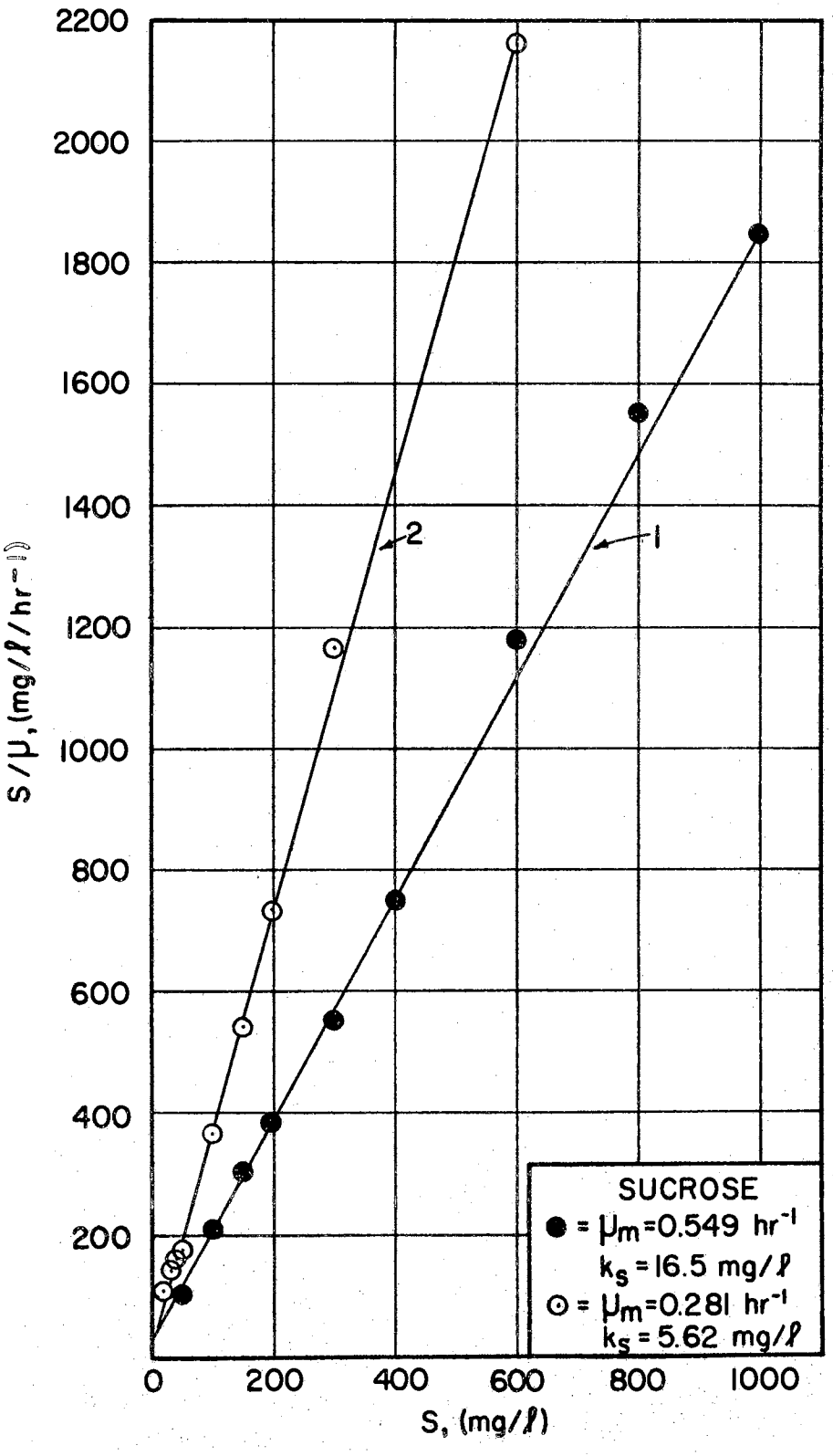

Figure 6. Lineweaver, Burk Plots for Sucrose

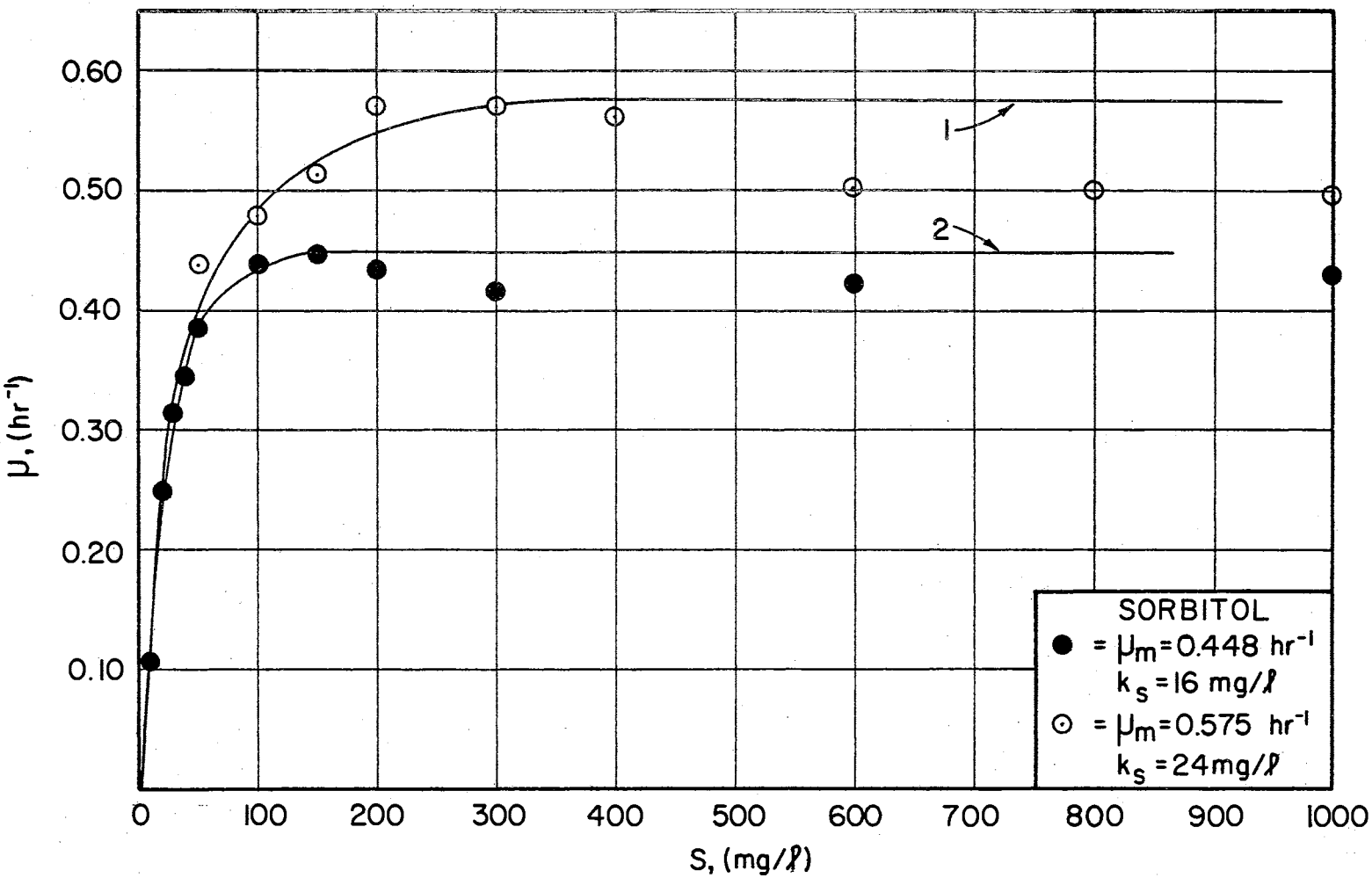

Figure 7. Monod Plots for Sorbitol

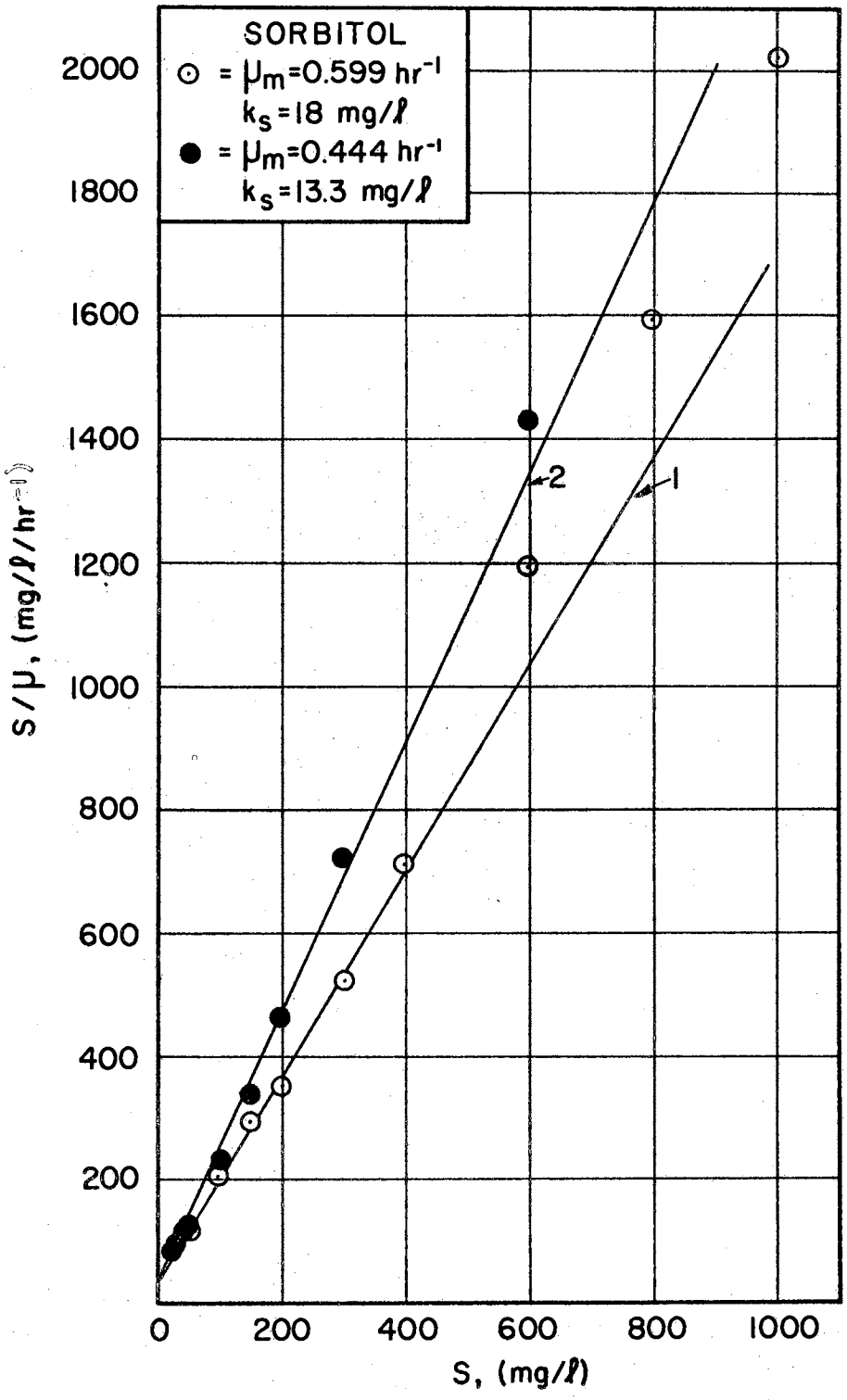

Figure 8. Lineweaver, Burk Plots for Sorbitol

in μ at the higher concentrations is evident in figure 7 and 8 for the first run.

B. Amino Acids

One amino acid from each of the six main groups was chosen as a carbon source. An acclimated population developed rapidly from a sewage seed for all amino acids studied with the exception of cysteine. With this substrate considerable difficulty was experienced. The alanine, serine, glutamic acid, and phenylalanine used were DL mixtures, while the L-cysteine and L-histidine were employed.

1. Alanine

Figures 9 and 10 show the results for the two experiments with alanine. At first the cells grew with a light flocculant appearance and the mixed liquor was a greenish color, but after seven days dispersed cells existed and the color was milky. At this time the first experiment was run; a 2 hour lag phase was observed, and as figure 9 indicates the values of μ for the low substrate concentrations were grouped at approximately the same growth rate. The cells in the stock culture system remained dispersed but the color of the mixed liquor changed again to green and remained this way during the second experiment. The growth rate was much lower with the greenish cells; the same effect as noted for the carbohydrate experiments. Both plots in figure 9 indicate a hyperbolic curve.

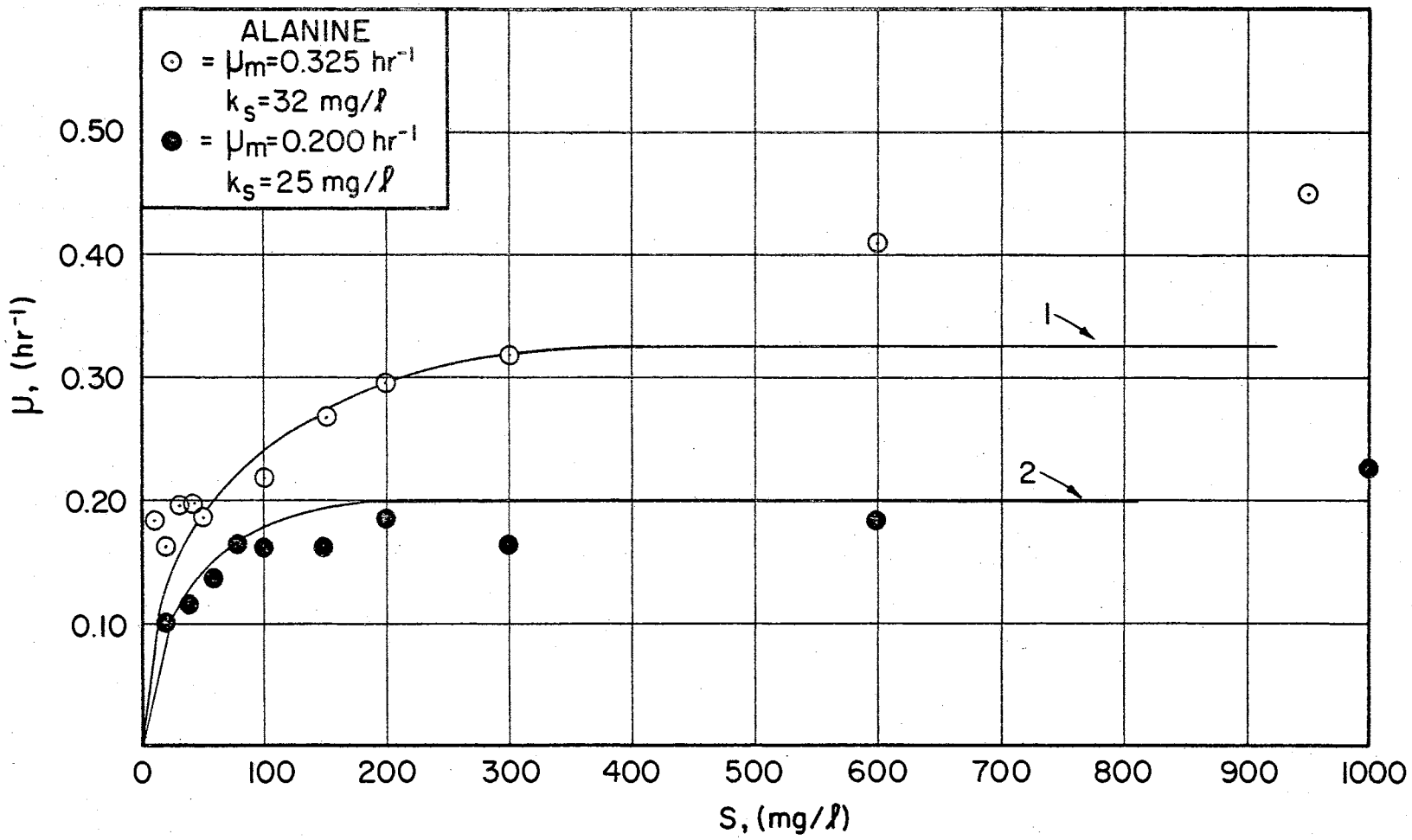

Figure 9. Monod Plots for Alanine

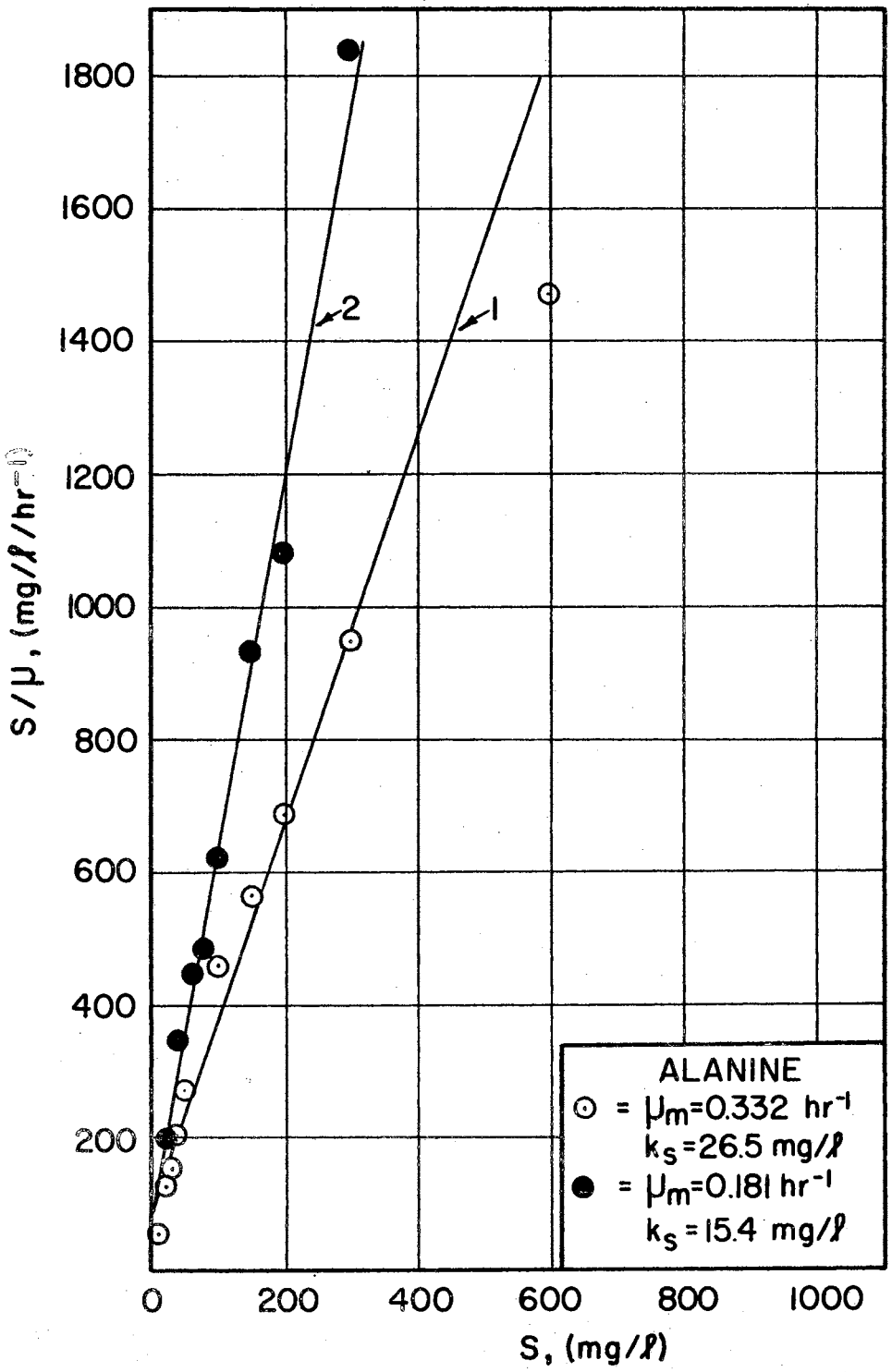

Figure 10. Lineweaver, Burk Plots for Alanine

2. Glutamic Acid

On glutamic acid the "stock" population exhibited at first a greenish color but after six days the mixed liquor was milky and the cells grew in a dispersed suspension; they remained this way during the period of running both growth experiments. The growth rate was very high in both runs. Figure 11 leaves little doubt that a hyperbola function described the relationship between μ and S.

3. Serine

The mixed liquor of the serine stock culture was pale green when the cells were used for the first run and was a light brown color at the time of the second run. The data of both experiments fit a hyperbolic curve rather well as shown in figure 13. A two hour lag phase (as in the case of alanine) was observed in both experiments.

4. Histidine

The results using histidine are shown in figures 15 and 16. There was no evidence of a gross change in predominant species in the stock population during the period of running experiments one and two. The mixed liquor was a brownish color. In experiment 1 the growth rate at the low concentrations was as fast as it was at the high concentrations of substrate. On the other hand in the second run there was a distinct decrease in μ at low substrate concentrations. As figure 15 indicates the hyperbolic

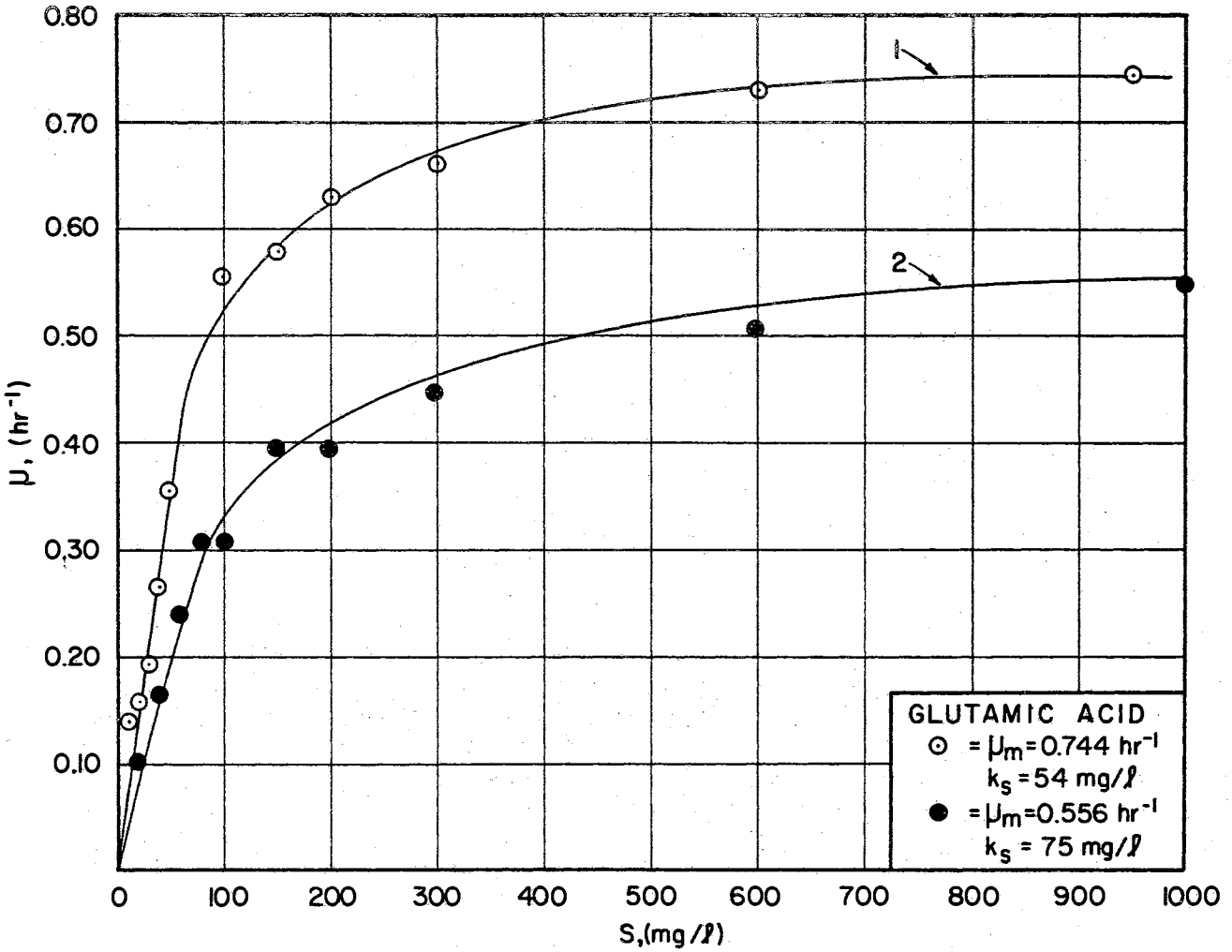

Figure 11. Monod Plots for Glutamic Acid

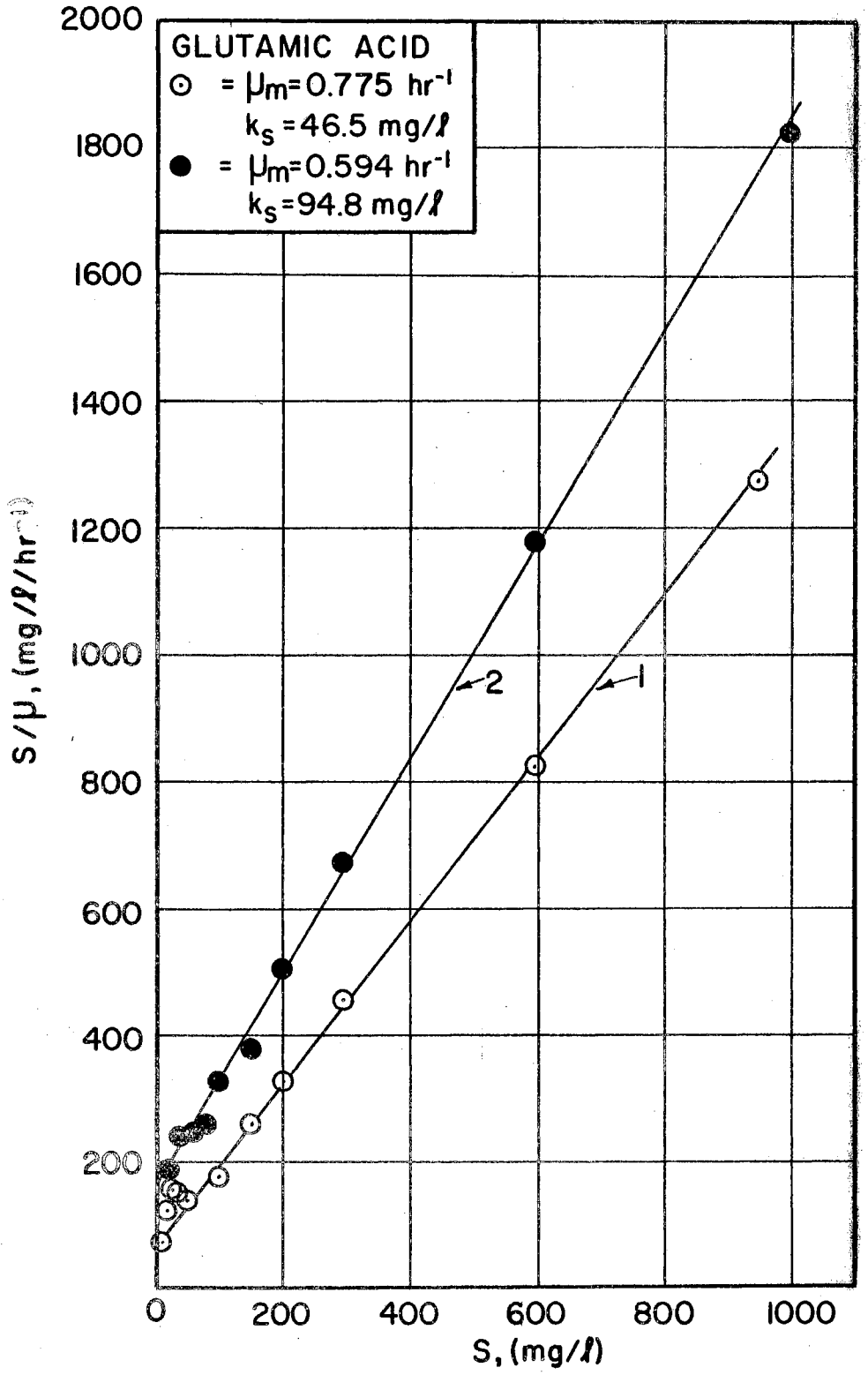

Figure 12. Lineweaver, Burk Plots for Glutamic Acid

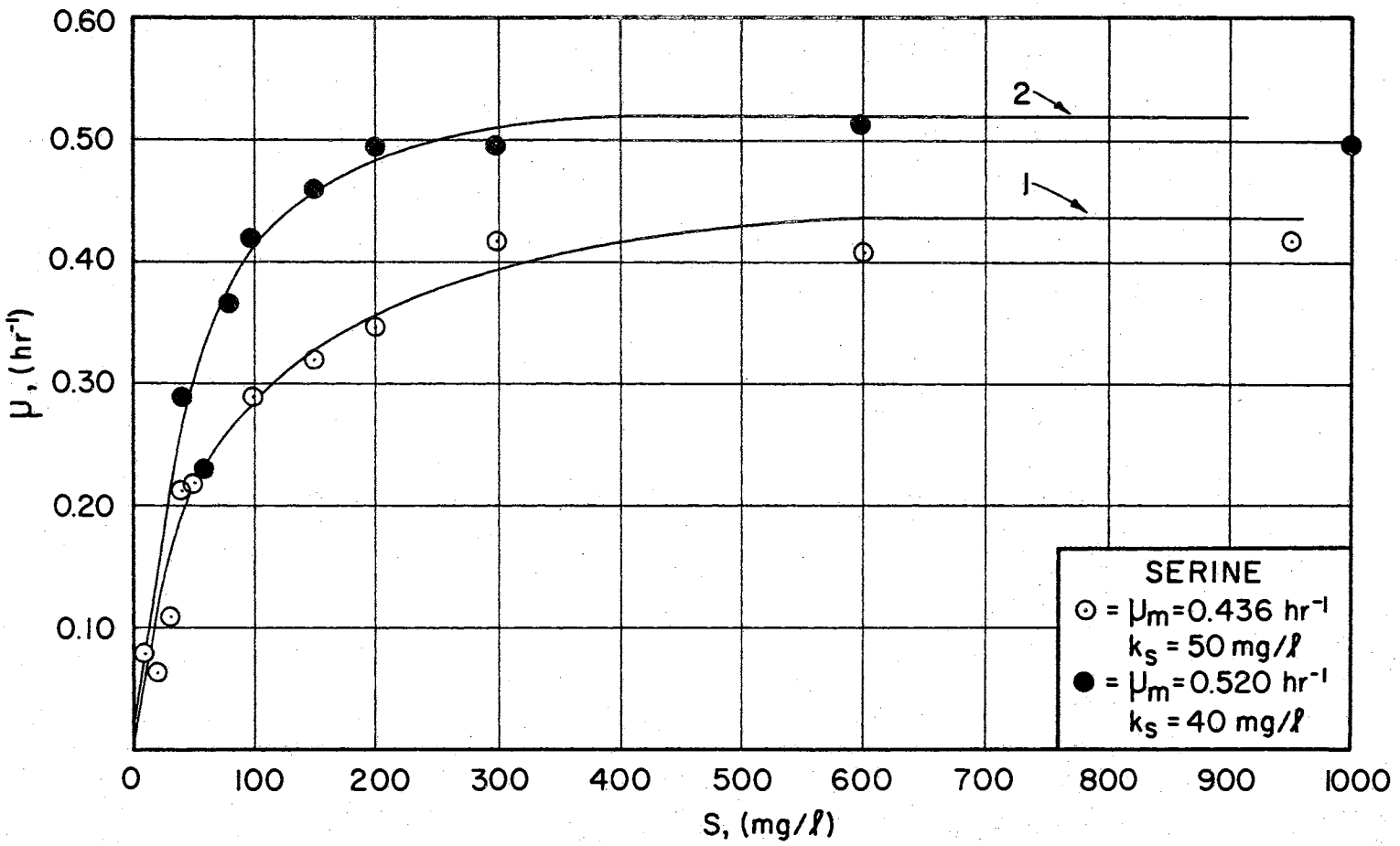

Figure 13. Monod Plots for Serine

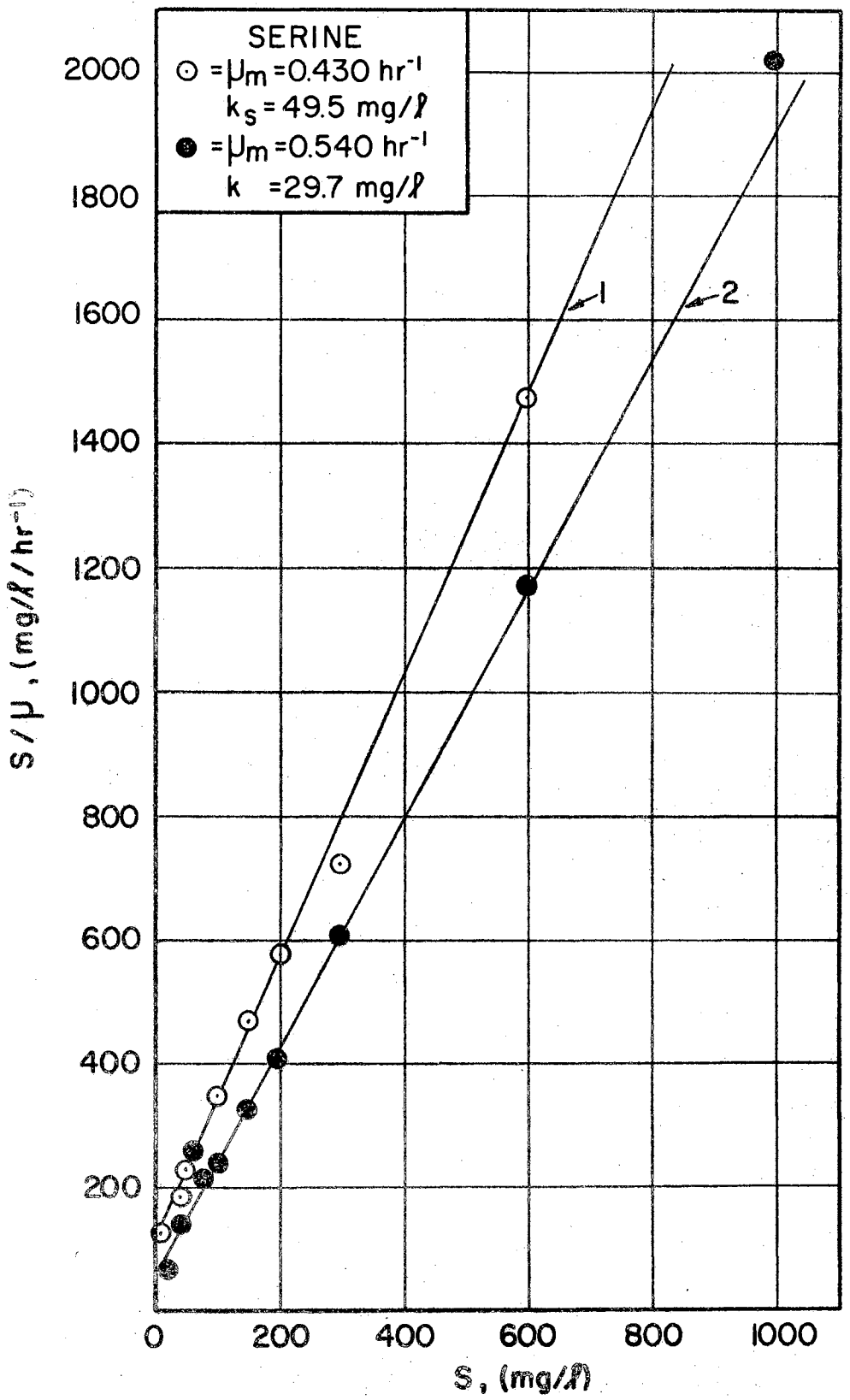

Figure 14. Lineweaver, Burk Plots for Serine

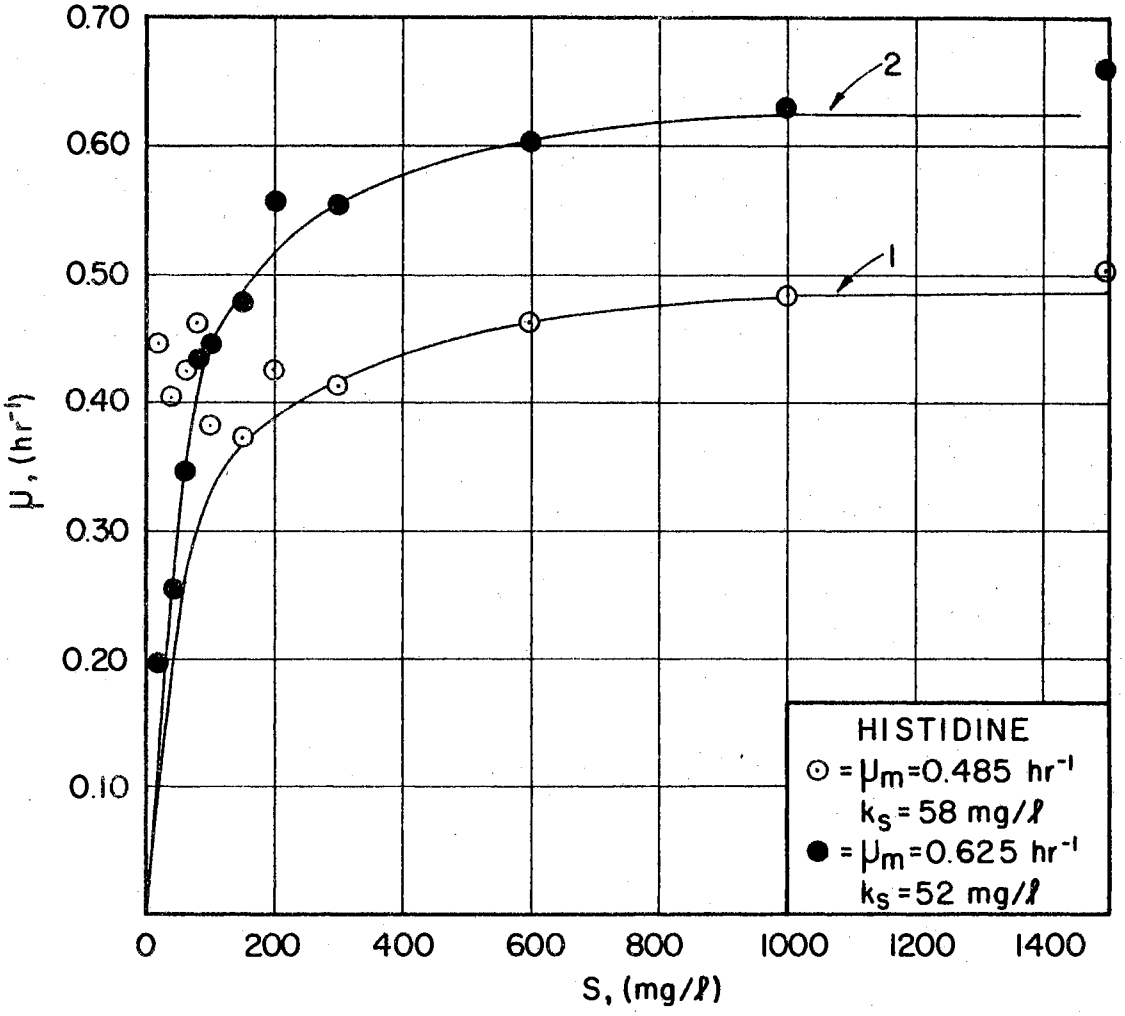

Figure 15. Monod Plots for Histidine

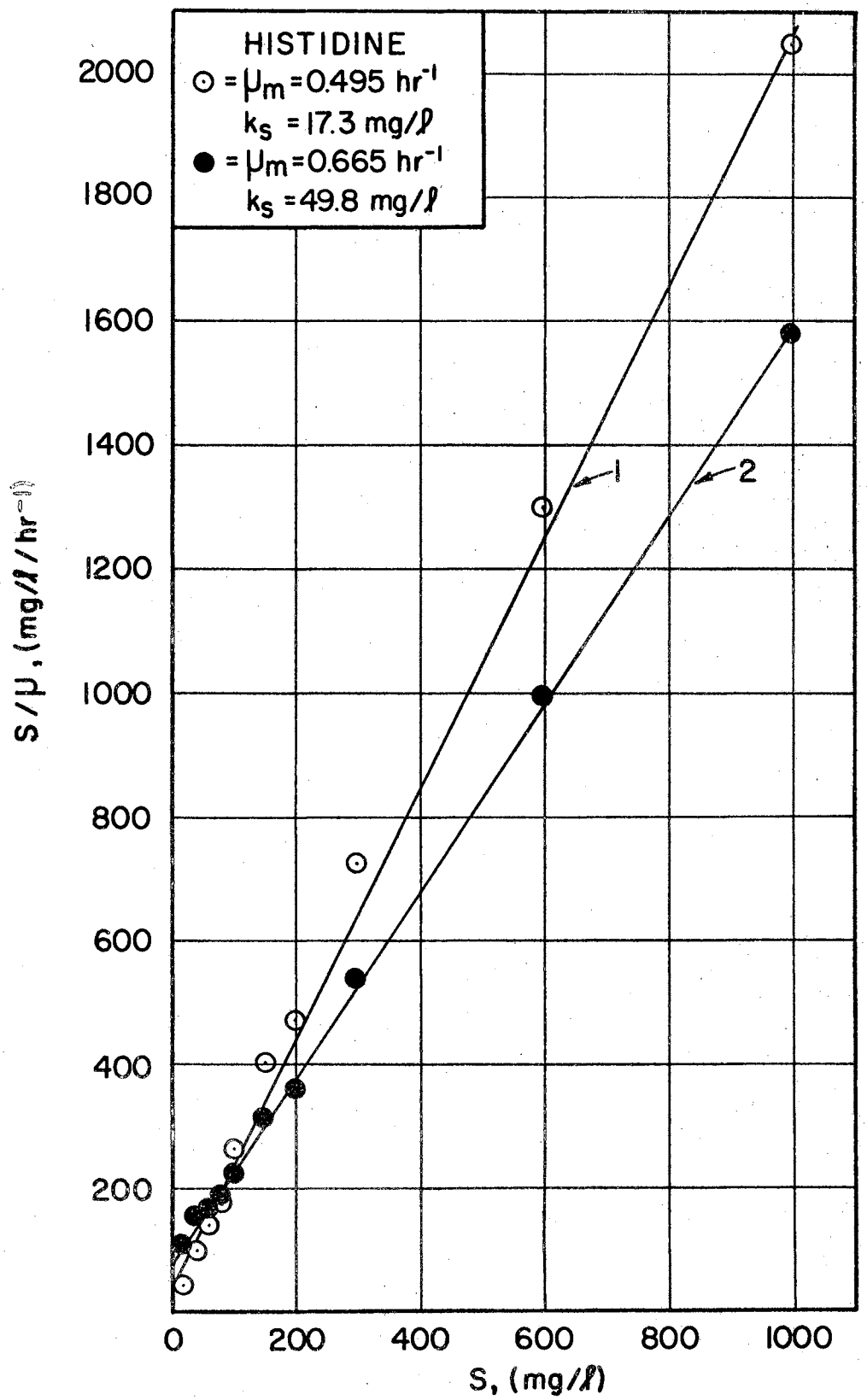

Figure 16. Lineweaver, Burk Plots for Histidine

curve was very much in evidence and the values for μ_m and k_s calculated for figure 15 and 16 agreed very well.

5. Phenylalanine

Figures 17 and 18 show the results for two growth rate experiments on phenylalanine. The mixed liquor of the stock culture had a milky appearance throughout the experimental period and both runs gave essentially the same results.

6. Cysteine

Figures 19 and 20 show only one experiment because the difficulty involved in working with cysteine discouraged further study of this substrate. A precipitate settled out of the stock feed solution and the odor of the feed was offensive and that of the mixed liquor during growth was worse. Also extra buffer was needed to maintain a neutral pH. The predominant cells imparted a distinctly green color to the mixed liquor. Growth was not abundant on the substrate and it was necessary to transfer three times the amount of cells into the experimental flasks in order to start a growth experiment. The growth rates were very low and difficult to determine. The growth in the low and medium range concentrations was dispersed, but in the flasks containing 600, 1000, and 1500 mg/l cysteine a flaky biomass was produced which rendered false transmittance readings. The results are considered to provide only a rough indication of the growth rates.

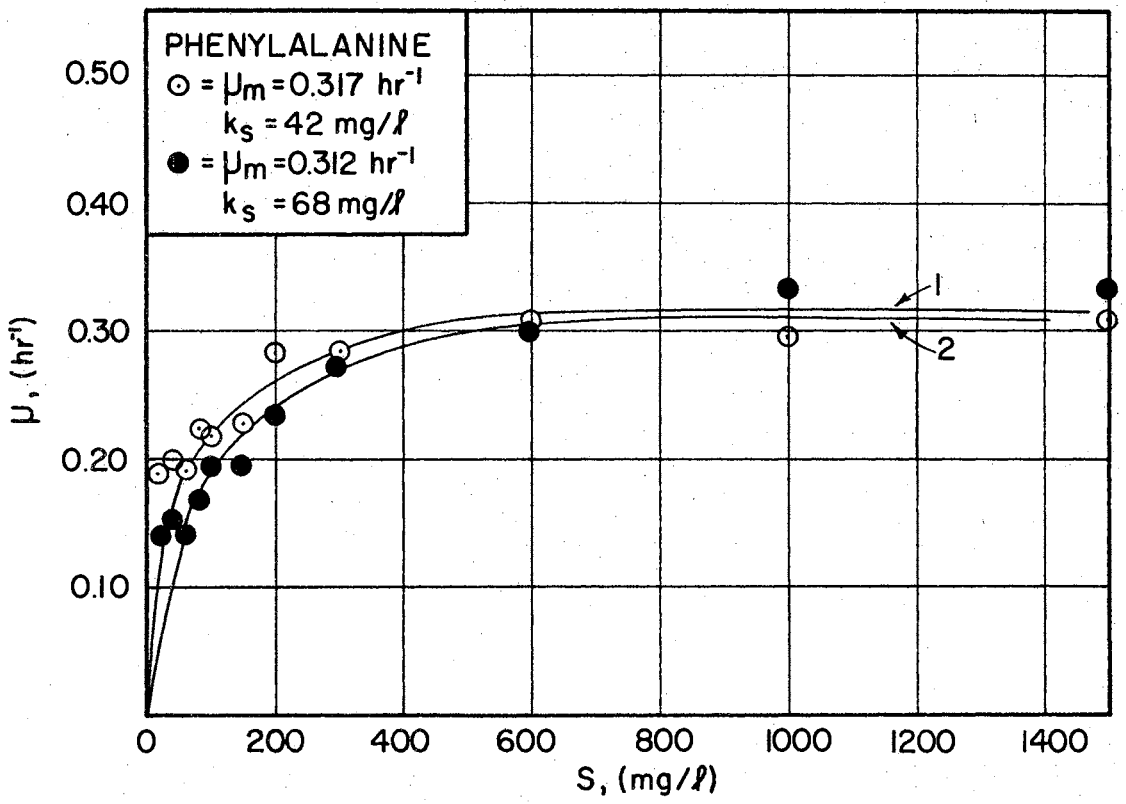

Figure 17. Monod Plots for Phenylalanine

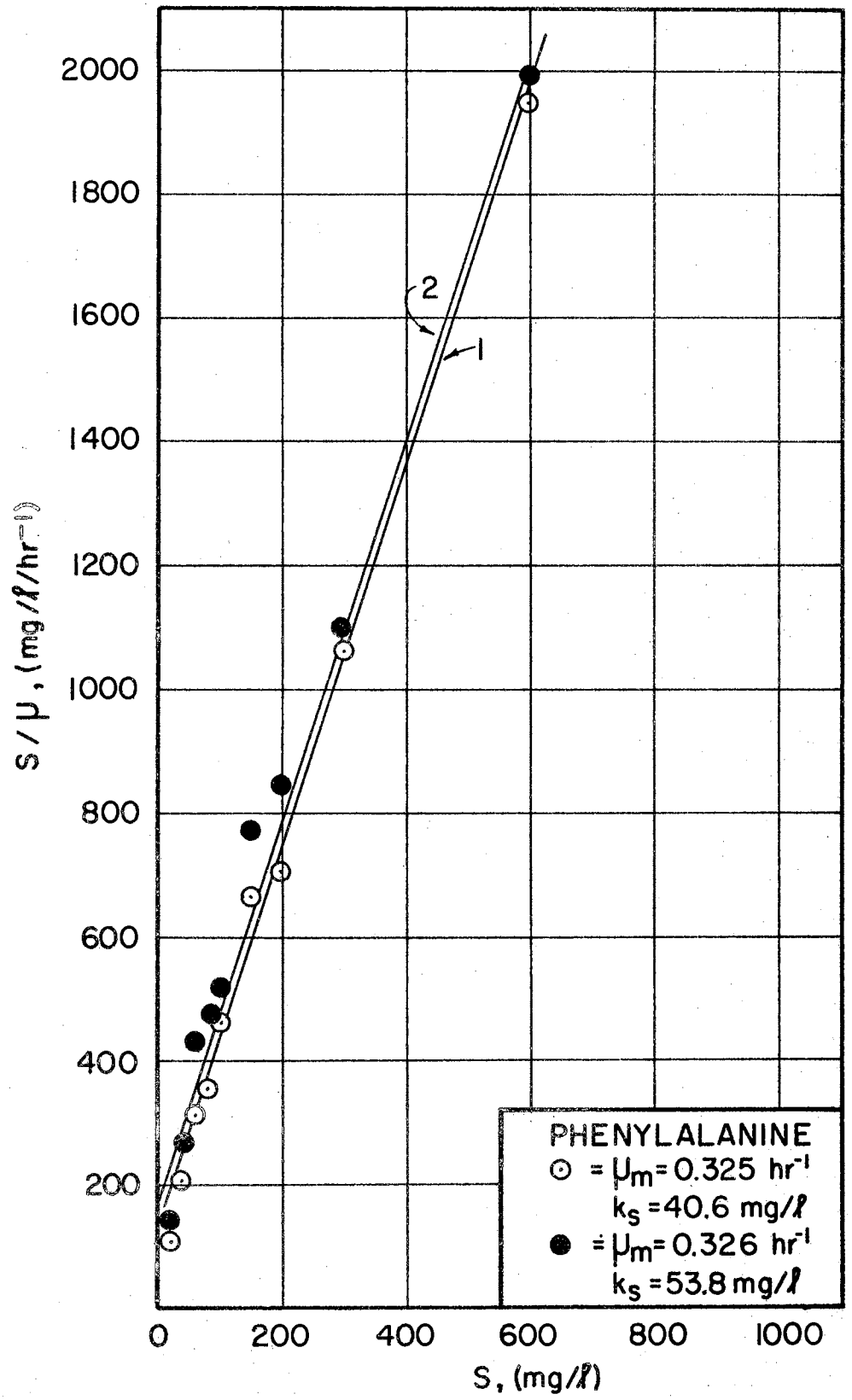

Figure 18. Lineweaver, Burk Plots for Phenylalanine

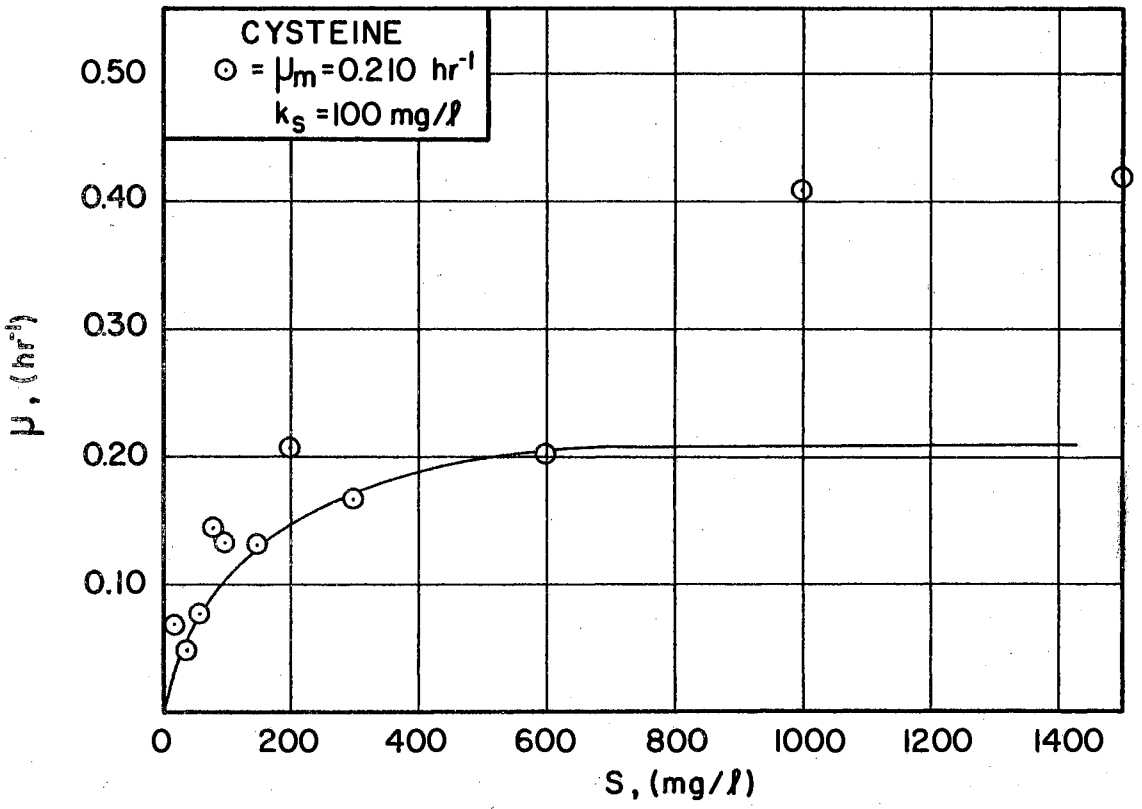

Figure 19. Monod Plot for Cysteine

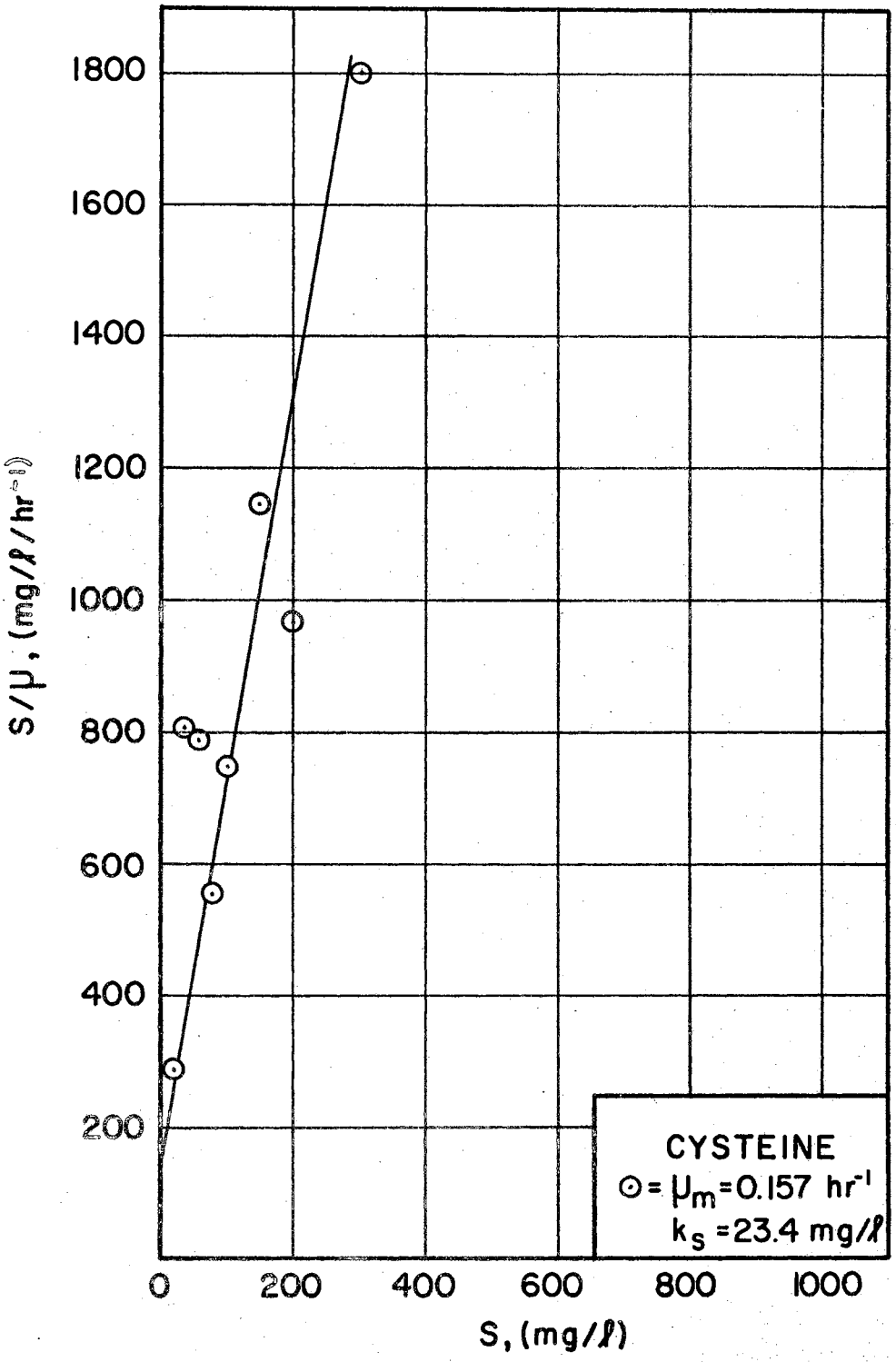

Figure 20. Lineweaver, Burk Plot for Cysteine

C. Fatty Acids

1. Acetic Acid

The results for growth on sodium acetate are shown in figure 21 and 22. No extra buffer was needed, and the pH remained at approximately seven during the experiments. Growth on this substrate imparted a green color to the mixed liquor which persisted during the entire experimental period. For experiment 1 substrate concentrations from 20-200 mg/l yielded approximately the same growth rate.

2. Propionic Acid

Figure 23 and 24 show the results for growth on propionic acid. No salt of the acid was available and extra buffer was added to hold the pH at seven. Initiation of growth was rather slow but after a few days a population developed and the mixed liquor had the same appearance as the acetic acid system. The first experiment on propionic acid was also like the first one for acetic acid in that the 20-300 mg/l substrate concentration yielded approximately the same growth rates. In any event it can be stated that there was considerable scatter in μ values in this substrate range and a curve in figure 23 was difficult to construct. The growth rate data for the second run indicated a much greater spread in this substrate range.

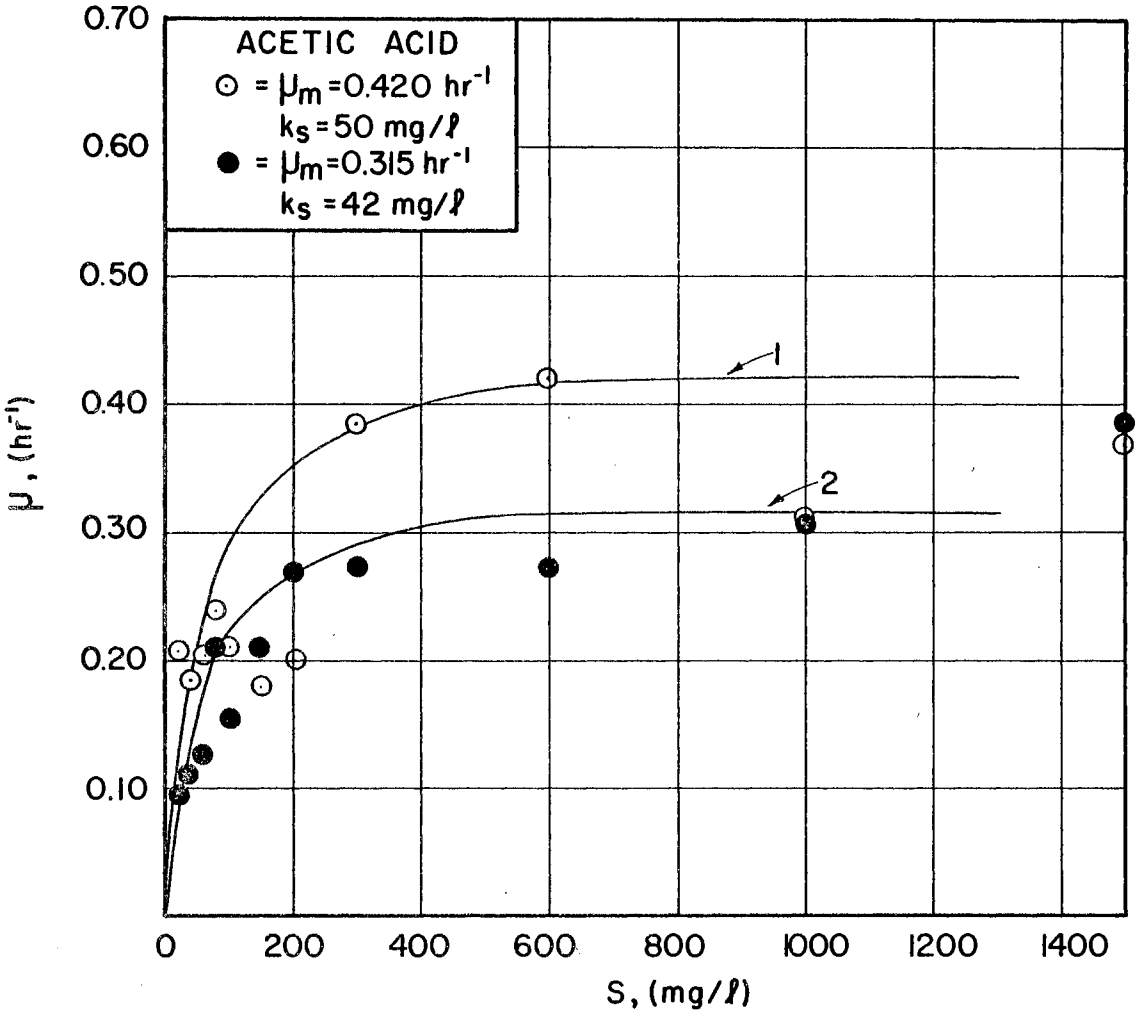

Figure 21. Monod Plots for Acetic Acid

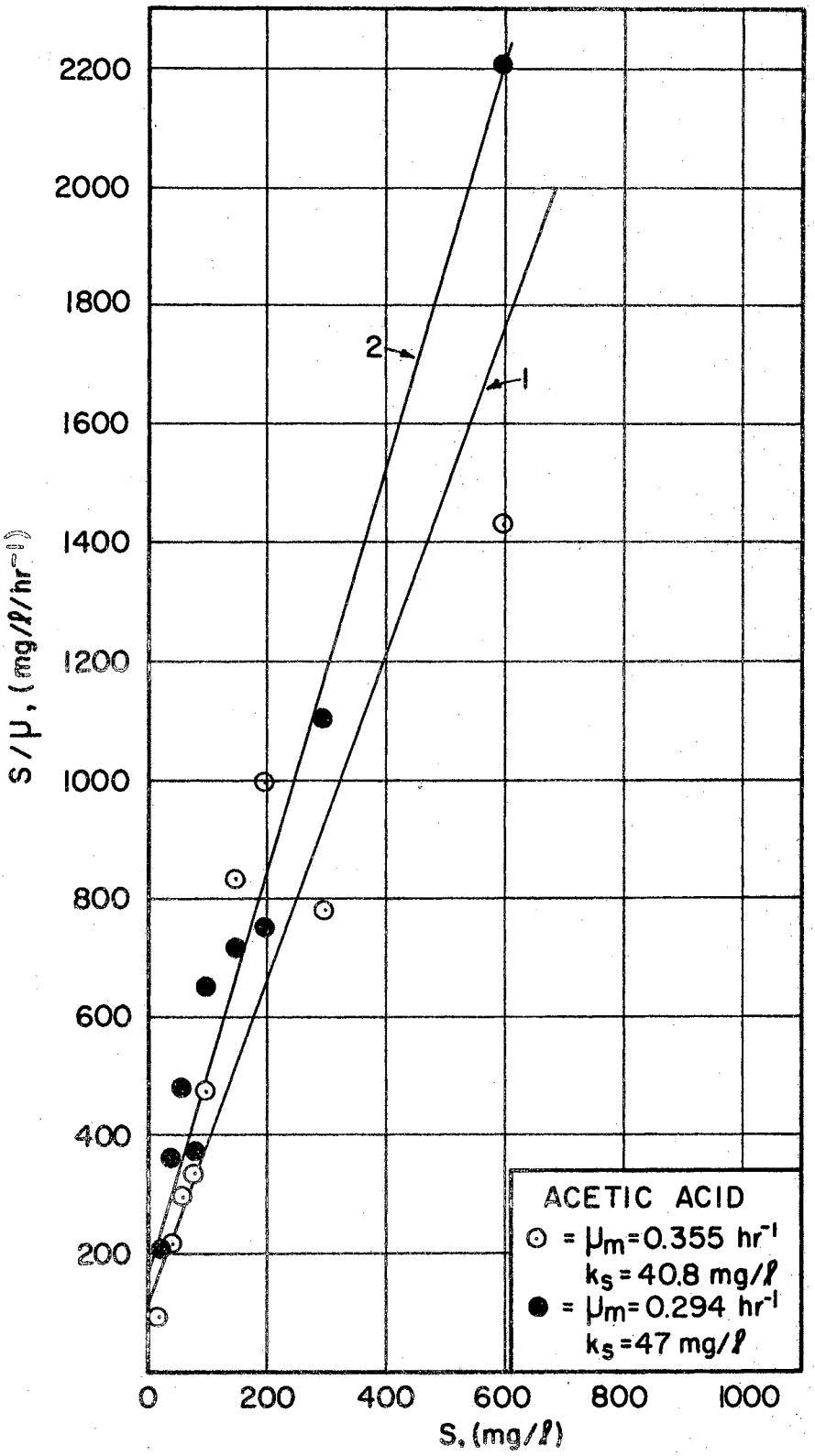

Figure 22. Lineweaver, Burk Plots for Acetic Acid

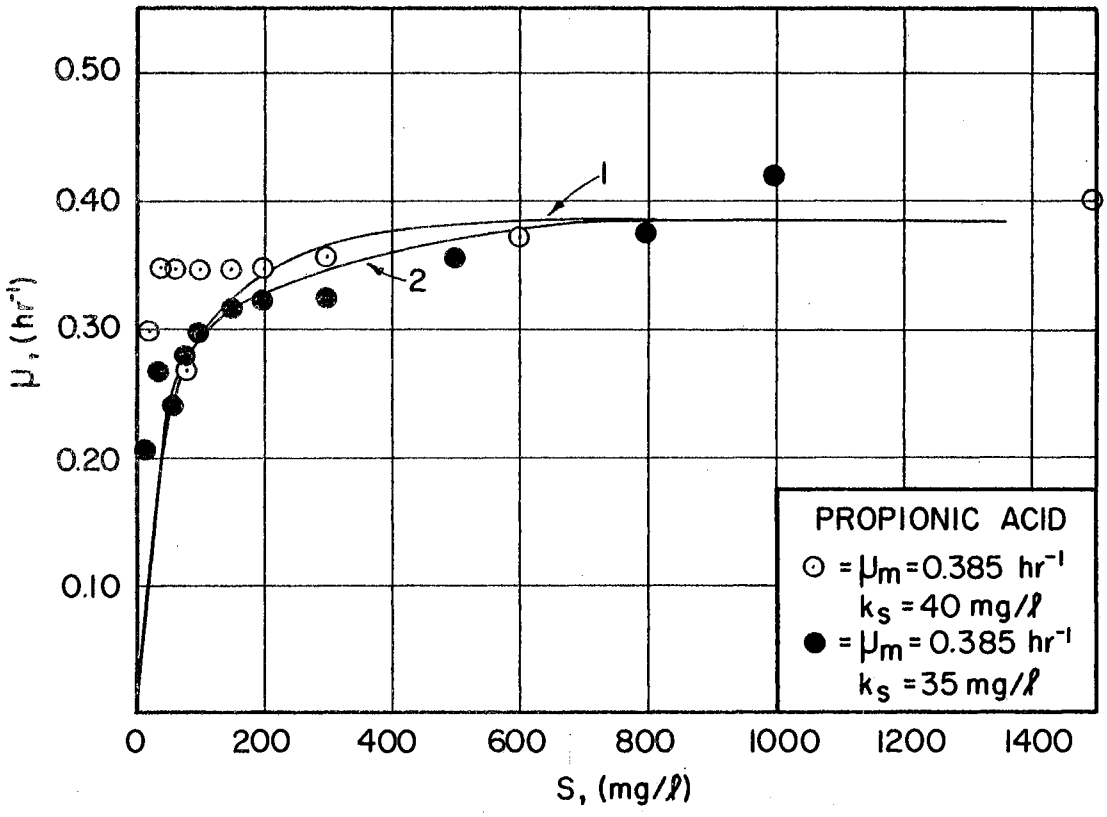

Figure 23. Monod Plots for Propionic Acid

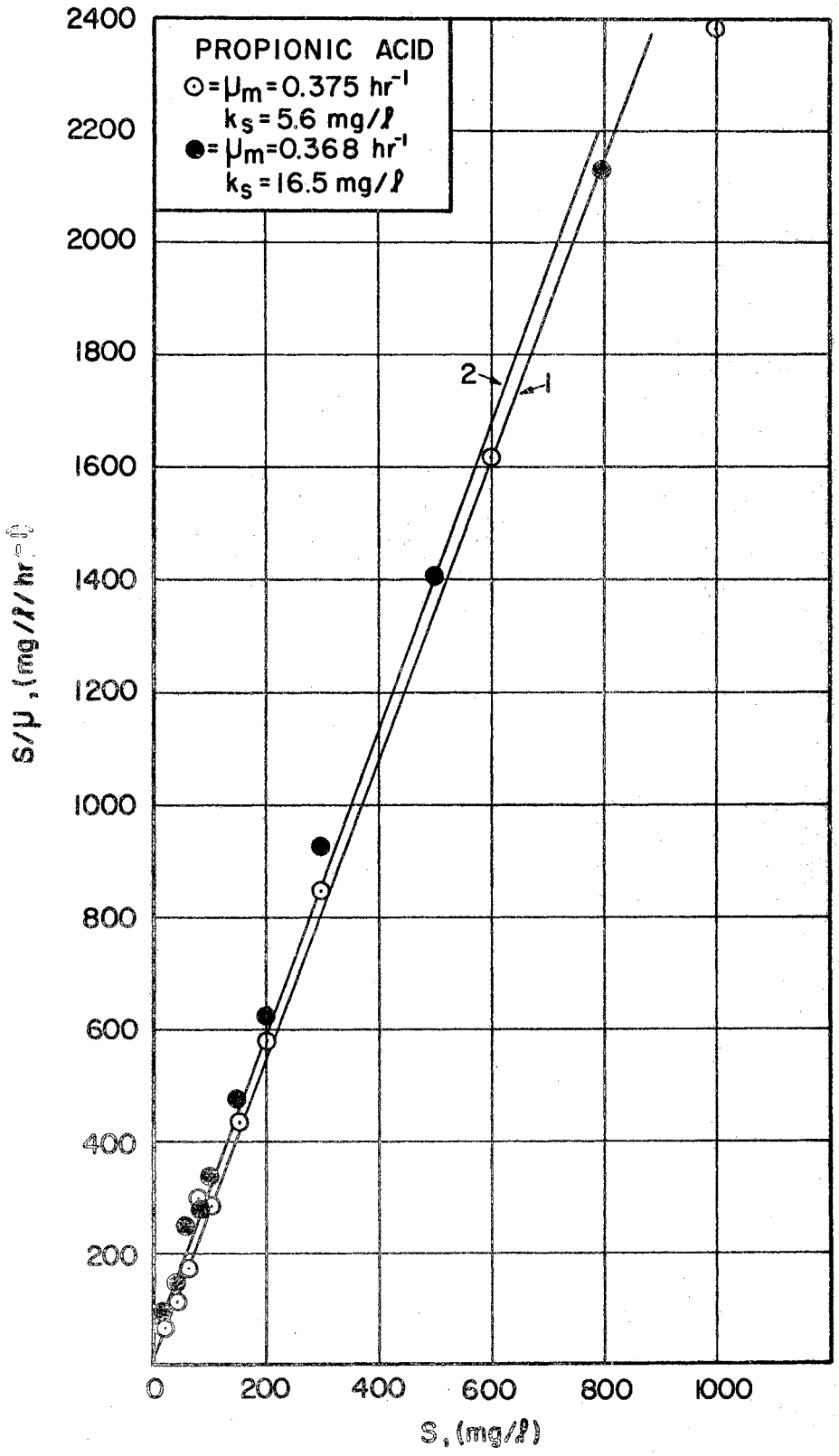

Figure 24. Lineweaver, Burk Plots for Propionic Acid

D. Municipal Sewage

The final substrate was municipal sewage from the Stillwater, Oklahoma treatment plant. The COD of the effluent from the primary clarifier was found to vary from 150 to 400 mg/l. When this waste was filtered (membrane filter 0.45 μ) the COD was reduced to ½ its value. In order to use the primary effluent as a substrate in the growth rate experiment it was necessary to concentrate it approximately twenty fold (500 ml portions evaporated to a volume of 25 ml). Figure 25 shows the COD value obtained when the sewage was concentrated at 65°C. The dotted lines represent the theoretical COD concentration if no COD were lost during the evaporation procedure. The solid lines are drawn through the observed COD's at various concentration ratios. The curves labeled "filtered" were obtained by filtering a portion of the same sample taken for the "unfiltered" results. Therefore, the unfiltered solid line is the amount of COD remaining in the concentrates of the primary effluent and the "filtered" solid line is the amount of soluble COD remaining in the concentrates. Filtered primary effluent was not concentrated. A stock solution of filtered concentrate (20:1) was used as the substrate for the first sewage experiment.

Figure 26 shows the results when another sample of primary effluent was concentrated at 55°C. Comparison of this figure with figure 25 indicates that the decrease in temperature increased the amount of COD remaining in the

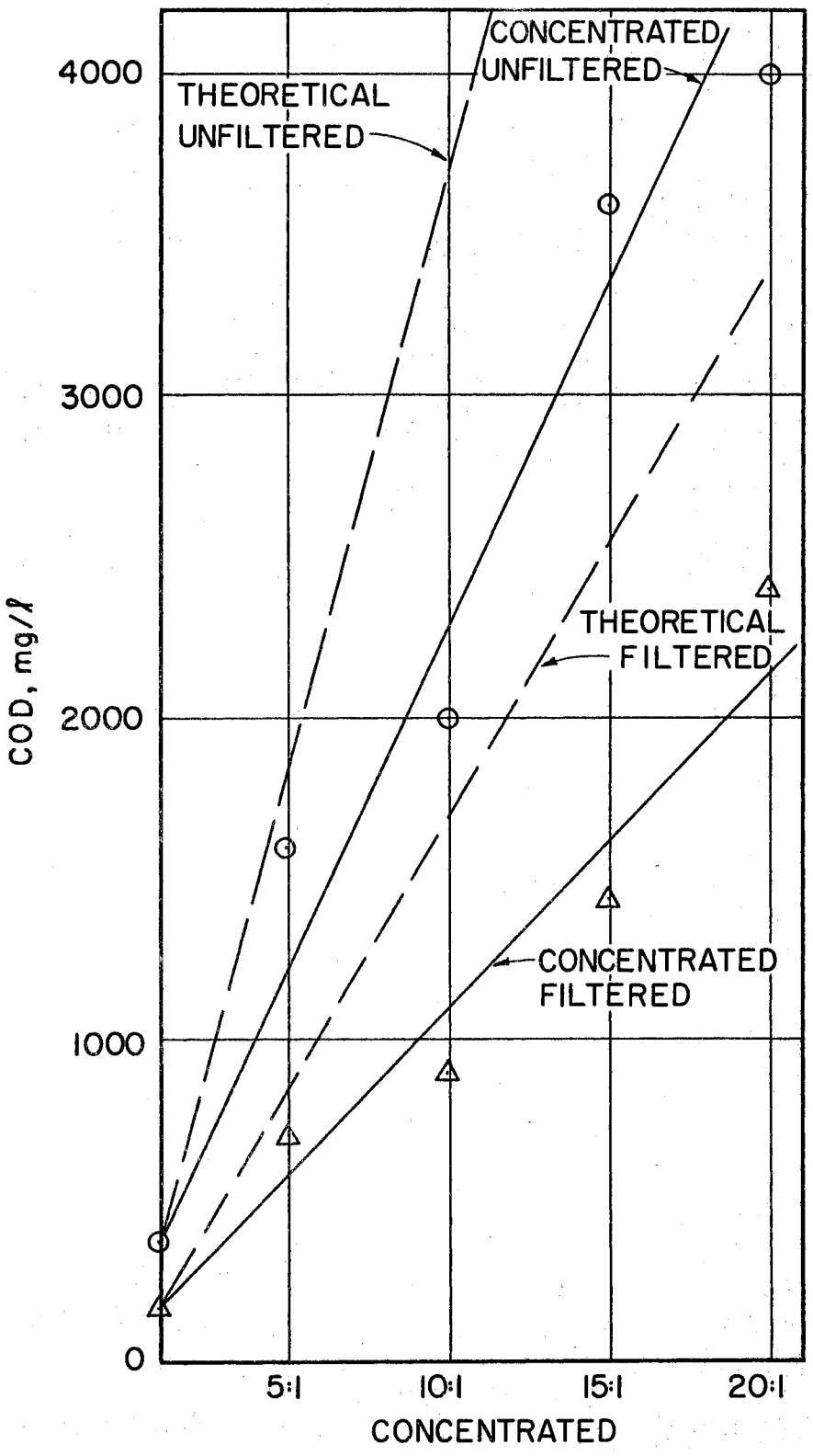

Figure 25. Effect of Concentrating Sewage at 65°C

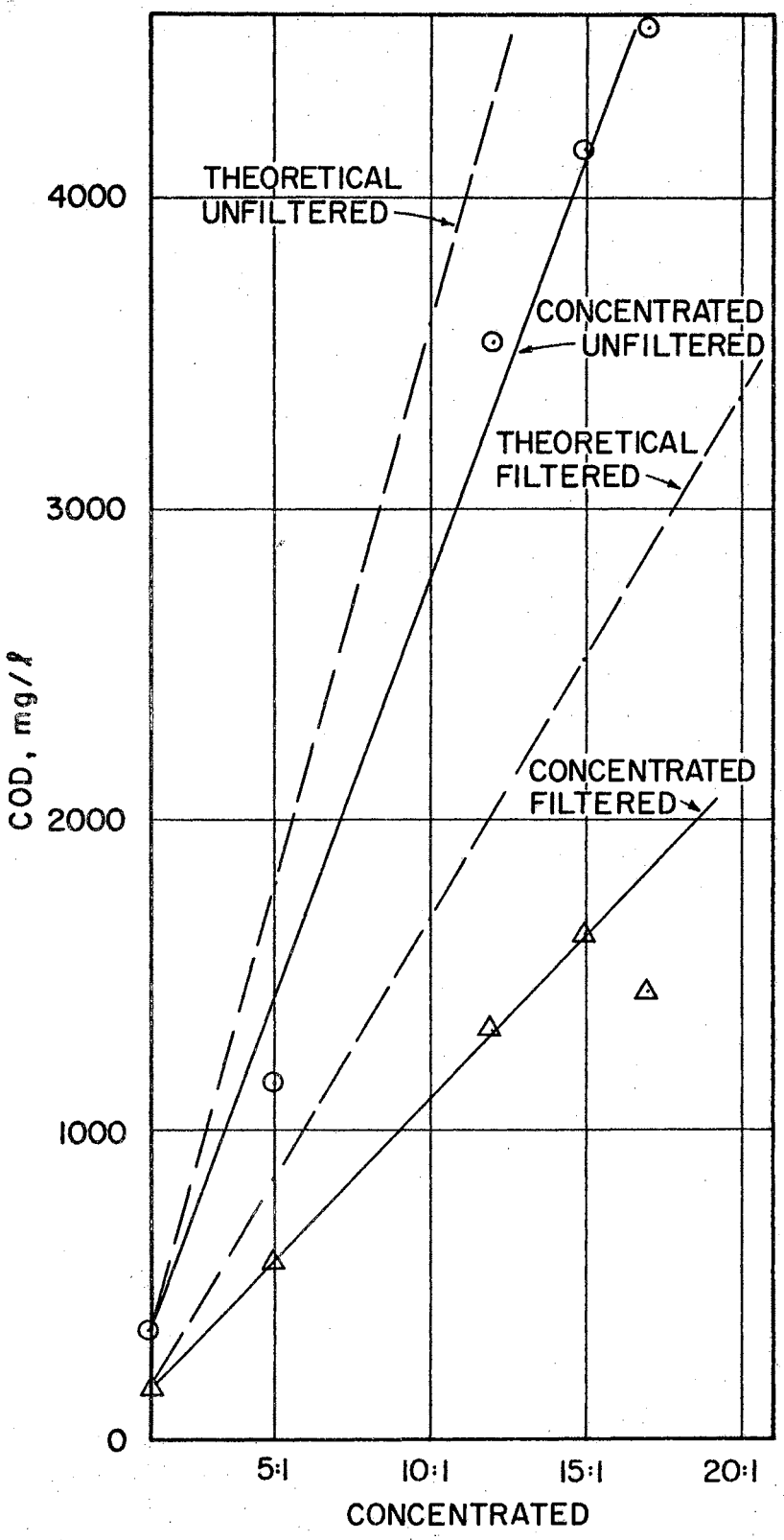

Figure 26. Effect of Concentrating Sewage at 55°C

concentrate. However, it is emphasized that different samples of primary effluent were used in each case and municipal sewage is not of constant composition. Temperatures lower than 55°C were also tried, but the time required for evaporation increased to approximately three hours with temperatures of 50°C and less. At 55°C it took between 45 and 60 minutes to evaporate 500 ml to 25 ml; a stock solution of this filtered concentrate was employed in the second experiment. Figure 27 shows the resulting growth curves and the values for the growth rates for each concentration employed. It is noted that the medium consisted only of filtered concentrate of primary effluent, appropriately diluted with distilled water, i.e., no mineral salts or buffer were added. It can be seen from figure 27 that the initial optical density is higher at the higher substrate concentrations. This is not because more seeding cells were used; the effect was caused by the increased color due to the higher sewage concentration. The pH of the feed was 8.6 in all growth flasks and remained so throughout the experiment except in the flask containing 10 mg/l COD. In this flask it dropped to 7.5 by the end of the growth period.

In figure 28 values of μ are plotted versus the substrate concentration. Figure 29 is a plot of S/μ versus S. The values of μ_m and k_s from both plots were very similar.

Growth curves for the second run are shown in figure 30. The 12.5 and 25 mg/l concentrations did not grow well

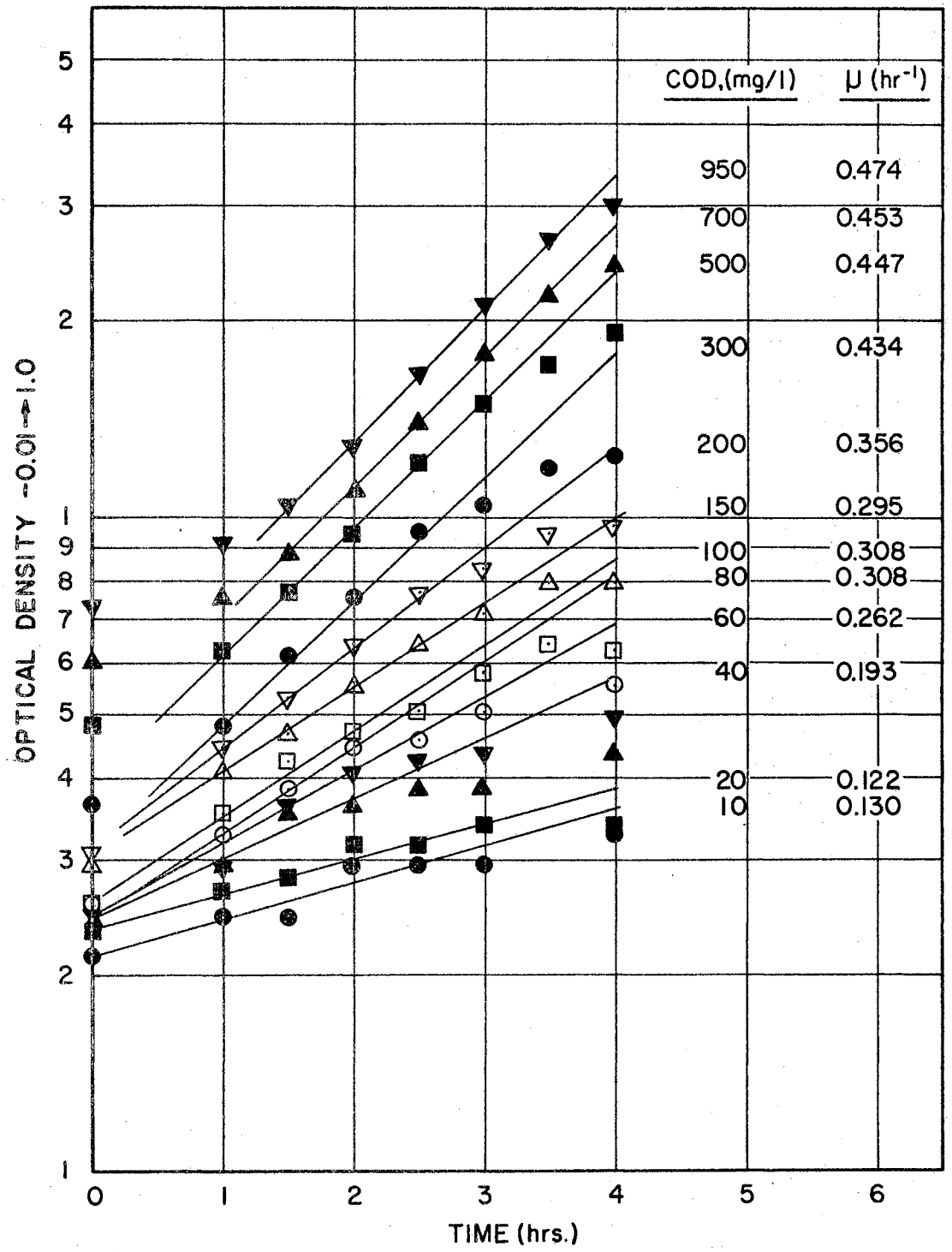

Figure 27. Growth Curves for Sewage at Various Concentrations for the First Run

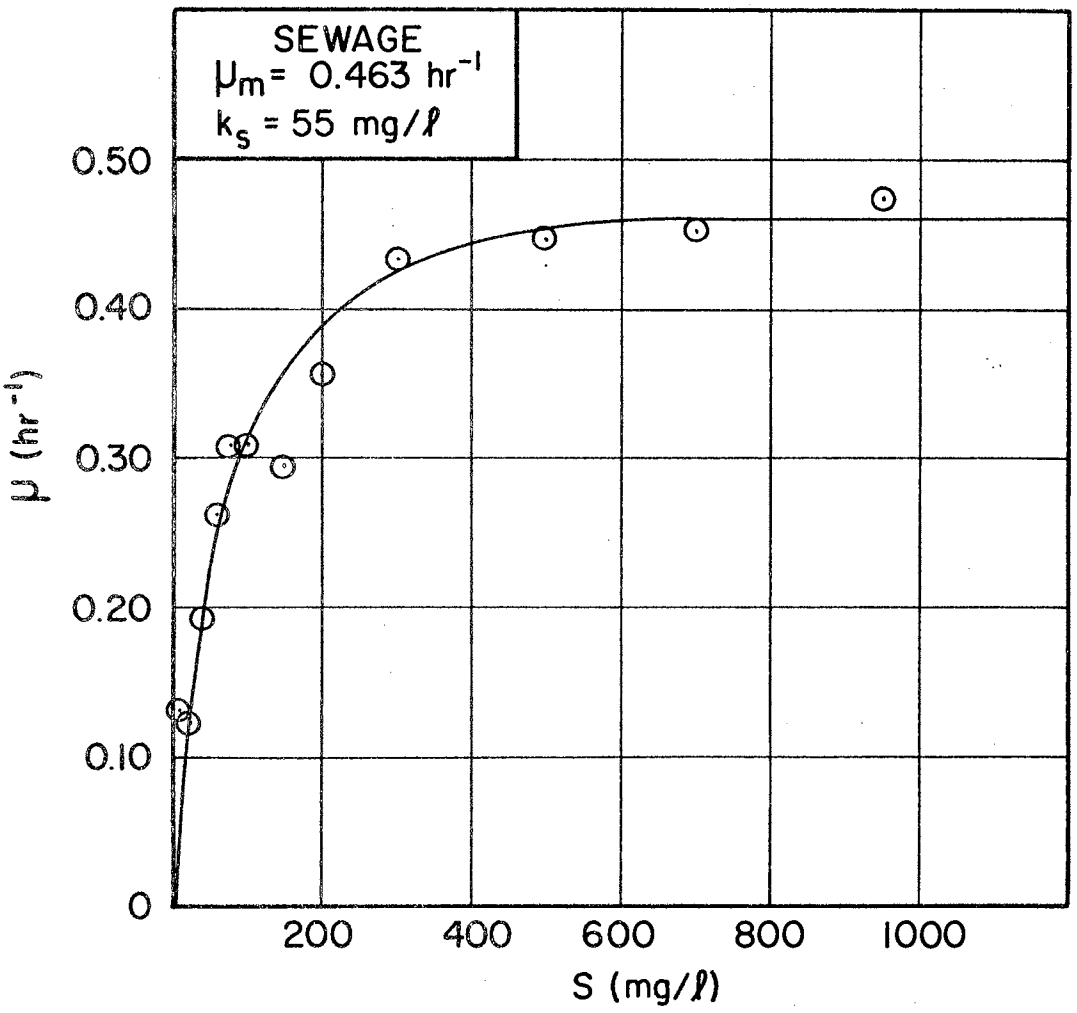

Figure 28. Monod Plot for the First Sewage Run

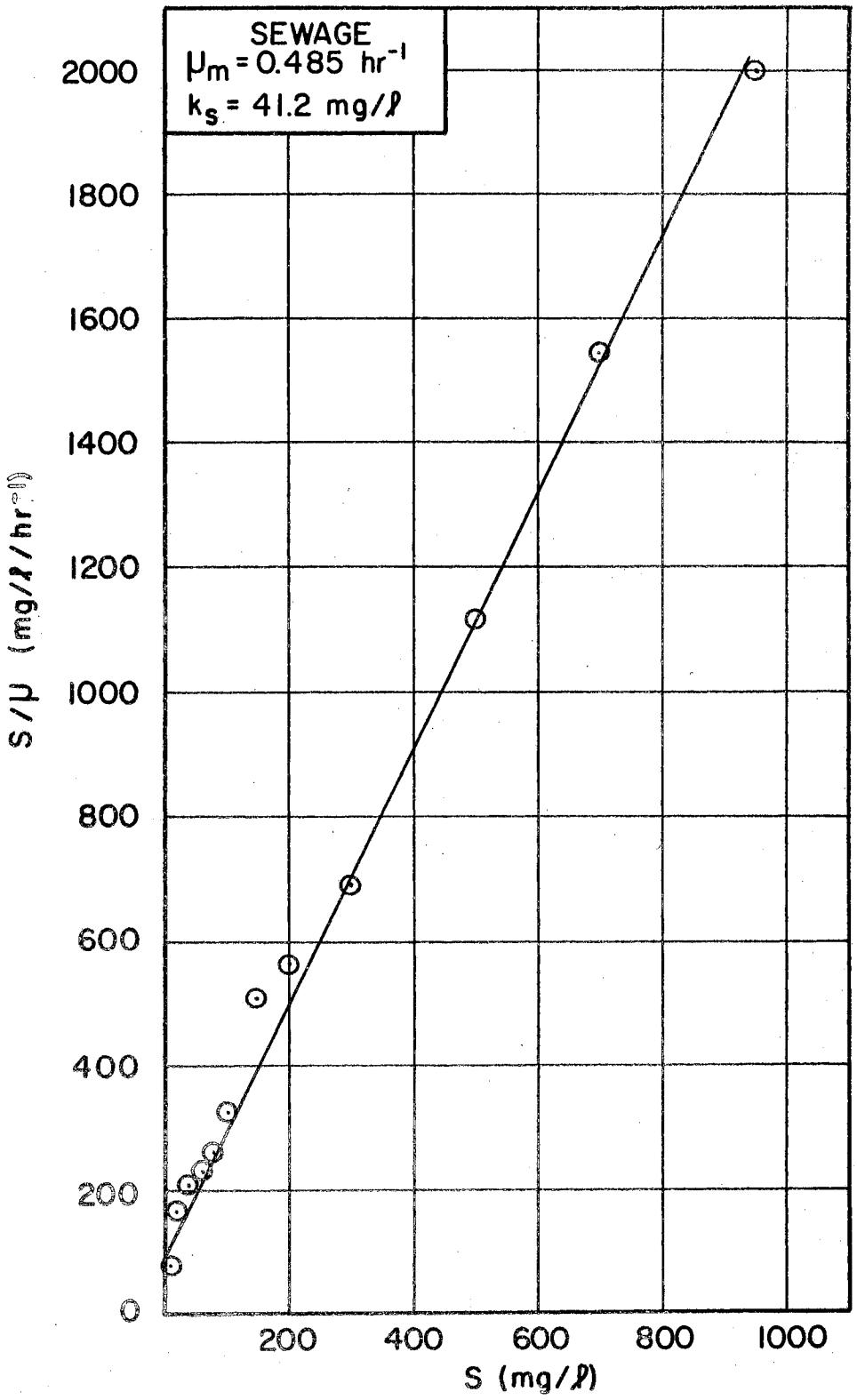

Figure 29. Lineweaver, Burk Plot for the First Sewage Run

and are not shown on the graph. This second batch of concentrated sewage yielded a slightly higher COD than the first. Growth at 1000 mg/l and 1300 mg/l COD yield lower values of μ than growth of the 700 mg/l COD level. The pH during this experiment was 8.5 as for the first run.

Figures 31 and 32 show the relationship between the log growth rate constant and COD concentrations. The values for μ_m and k_s are similar to those for the first experiment using sewage concentrate.

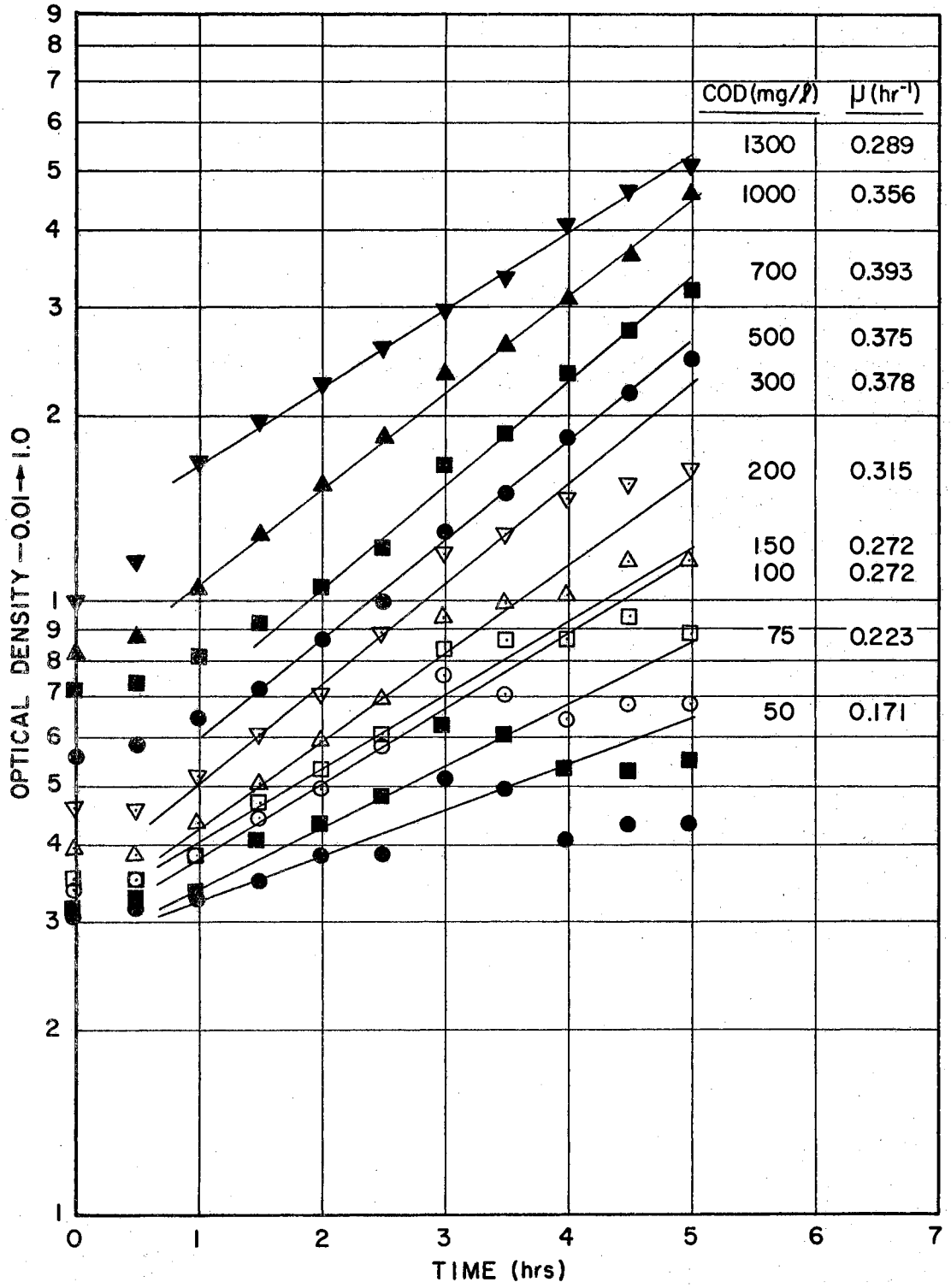

Figure 30. Growth Curves for Sewage at Various Concentrations for the Second Run

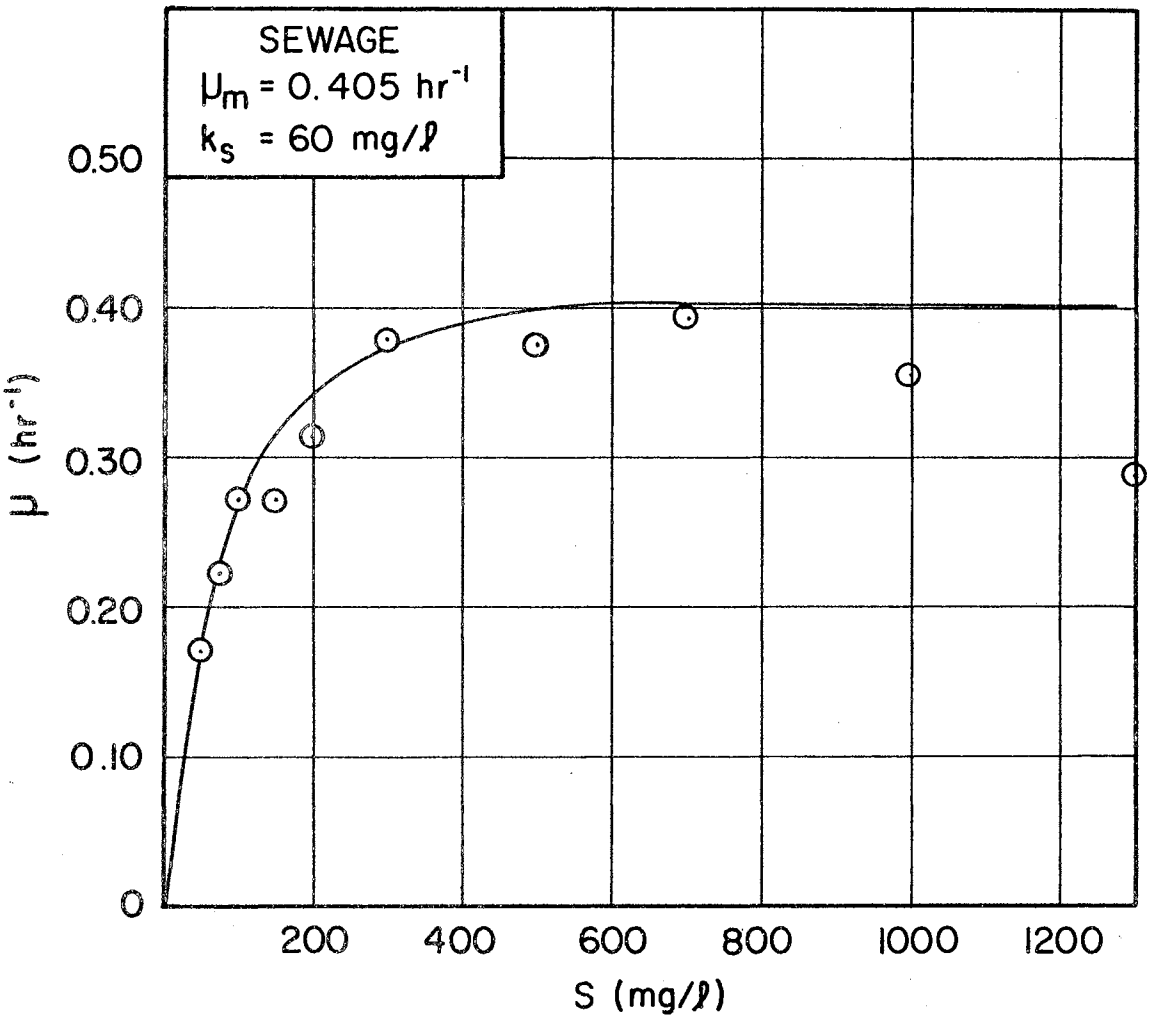

Figure 31. Monod Plot for the Second Sewage Run

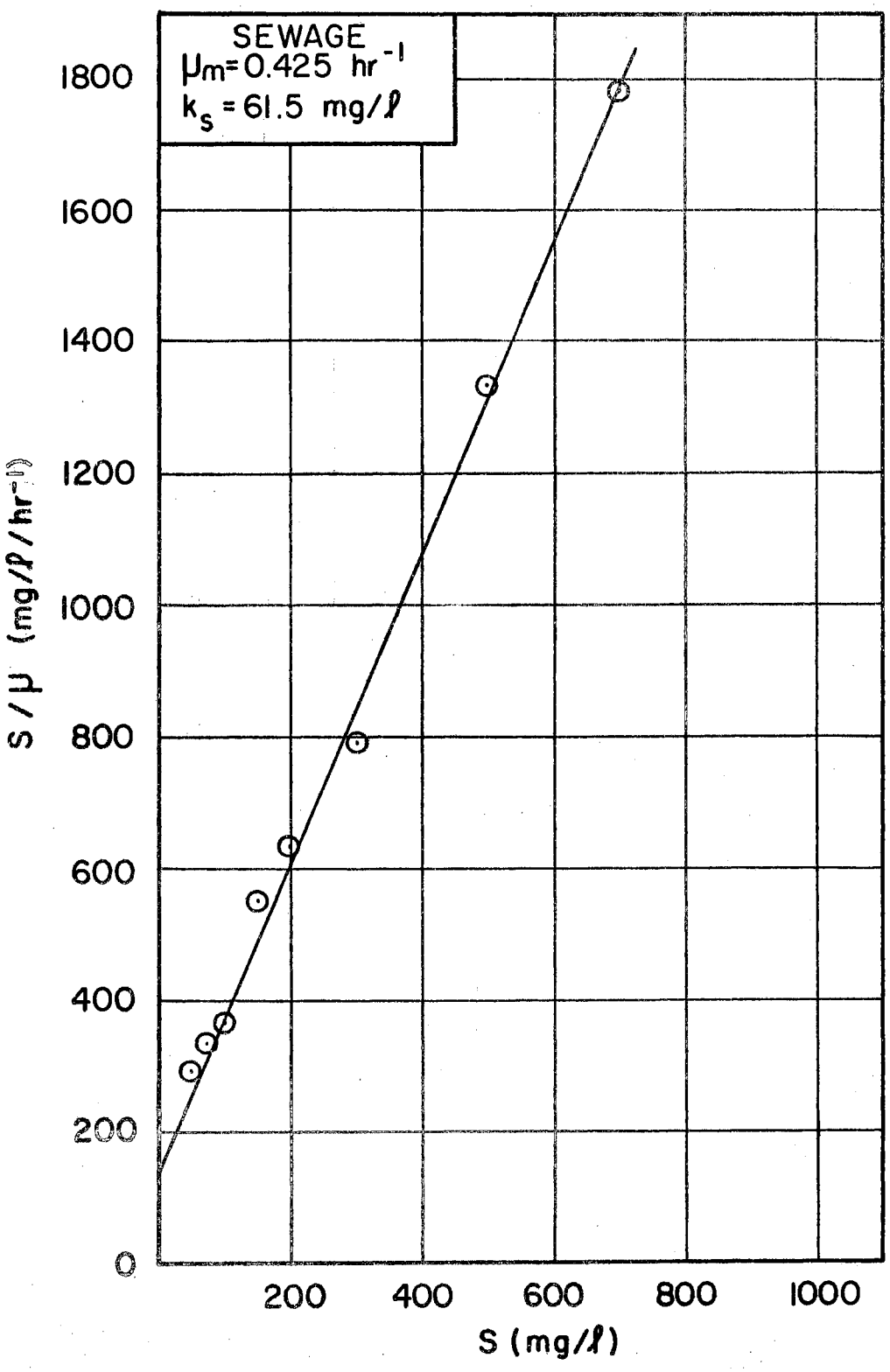

Figure 32. Lineweaver, Burk Plot for the Second Sewage Run

TABLE II

VALUES OF μ_m AND k_s FOR HETEOGENEOUS POPULATIONS GROWING ON VARIOUS SUBSTRATES

Substrate	Figure No.	Expt. No.	Lineweaver, Burk Plot (S/μ vs S)		Monod Plot (μ vs. S)		Hyperbolic Curve	Two Phase	Indeterminant
			$\mu_m(hr^{-1})$	k_s(mg/l)	$\mu_m(hr^{-1})$	k_s(mg/l)			
Glucose	1, 2	1	0.491	29.4	0.438	26			X
Glucose		2	0.380	11.4	0.380	10	X		
Lactose	3, 4	1	0.526	55.3	0.510	85	X		
Lactose		2	0.438	37.2	0.426	100	X		
Lactose		3	0.200	0.0	0.252	40			X
Lactose		4	0.434	32.5	0.417	35	X		
Sucrose	5, 6	1	0.549	16.5	0.545	17			X
Sucrose		2	0.281	5.6	0.290	12	X		
Sorbitol	7, 8	1	0.599	18.0	0.575	24	X		
Sorbitol		2	0.444	13.3	0.448	16	X		
Alanine	9, 10	1	0.332	26.5	0.325	32	X		
Alanine		2	0.181	15.4	0.200	25	X		
Glutamic Acid	11, 12	1	0.775	46.5	0.744	54	X		
Glutamic Acid		2	0.594	94.8	0.556	75	X		
Serine	13, 14	1	0.430	49.5	0.436	50	X		
Serine		2	0.540	29.7	0.520	40	X		
Histidine	15, 16	1	0.495	17.3	0.485	58			X
Histidine		2	0.665	49.8	0.625	52	X		
Phenylalanine	17, 18	1	0.325	40.6	0.317	42	X		
Phenylalanine		2	0.326	53.8	0.312	68	X		
Cysteine	19, 20	1	0.157	23.4	0.210	100			X
Acetic Acid	21, 22	1	0.355	40.8	0.420	50			X
Acetic Acid		2	0.294	47.0	0.315	42	X		
Propionic Acid	23, 24	1	0.375	5.6	0.385	40			X
Propionic Acid		2	0.368	16.5	0.385	35	X		
Sewage	28, 29	1	0.485	41.2	0.463	55	X		
Sewage	31, 32	2	0.425	61.5	0.405	60	X		

CHAPTER V

DISCUSSION

The purposes of this study were to determine whether the Monod relationship (3) was applicable to the variety of substrates tested, and if so to gain knowledge as to the range of values for μ_m and k_s which could be expected for heterogeneous populations developed from sewage. It may be concluded that, for all the substrates used, the Monod relationship provides a better fit of the data than does the linear two phase concept (4). This finding is in agreement with previous work done in this bioengineering laboratory on glucose (1) (2). The conclusion is also in agreement with the many authors who accept the Monod concept but modify the equation in various ways (6) (11-22).

Most of the values for μ_m and k_s cited in the literature review were for carbohydrates, and most of the experiments were done with pure cultures. Thus, it is not possible to compare directly the values of μ_m and k_s obtained with heterogeneous populations with those found in the literature. It is interesting to note the

three different values obtained for μ_m with glycerol as a substrate (6) (18) (22). In each study a different organism was employed and experimental conditions were different; the values for μ_m were 0.85, 0.506, and 0.45 hr^{-1}. Another common substrate was glucose, and values of 1.2, 0.42, 0.53, and 0.47 hr^{-1} were reported (20) (21) (1) (2). Again in each case the organisms were different.

The values obtained on glucose for the present study are similar to those reported by Gaudy, Ramanathan, and Rao (1); heterogeneous populations were used in both cases and similar growth conditions were employed. In the study of Gaudy, et al., the seed populations were grown under continuous flow conditions while all work in this study was carried out under batch conditions.

When the carbohydrate experiments were started, it was not known how low the concentrations should be; therefore, the first plot in the four carbohydrate experiments turned out to be incomplete for the low k_s values. Concentrations below 50 mg/l were then included; in some cases the data were improved and in others the growth was very irregular. In the experiments using glucose, sucrose, and sorbitol, the second runs were done with a green colored cell suspension, possibly a Pseudomonas and in each case the growth rate was lower than for the first run. On lactose a blue colored suspension developed after the flaky type growth was redispersed, which also lowered the growth rate in the third run. It was believed after the

final two runs that lactose would not fit the straight line Lineweaver, Burk plot, but the last two experiments did not indicate the linear two phase curve. It has been established that some bacteria need a specific enzyme system to break down lactose into galactose and glucose (24). This could be the reason for the longer lag.

In general, the four carbohydrates reacted in a similar manner. The results obtained in each case tend to support the Monod concept rather than a linear two phase relationship.

The cultures grown on amino acids differed in several characteristics. The glutamic acid and histidine units contained growth as heavy as the glucose system. The alanine, serine, and phenylalanine units maintained a greenish cell growth most of the time and was less turbid than for growth on glutamic and histidine. The cysteine unit never maintained a heavy growth at all. Where a change of predominance from the turbid milky color to a light green growth was evident the differences in μ_m were similar to those results for the carbohydrates. The green type of growth produced a lower growth rate. This was the case of the alanine and serine experiments.

Glutamic acid and histidine produced the two highest values for μ_m. On both substrates a green colored cell suspension persisted for a few days but the mixed liquor later changed to a very turbid milky white appearance for the glutamic acid and a brown color for the histidine system.

The phenylalanine experiments were the only ones to give almost exactly the same values for μ_m, however, the k_s values were slightly different between the two runs. The cells didn't seem to be able to use the substrate to any great extent; the higher concentrations didn't support much more growth than the lower concentration.

Work accomplished on amino acids by C. P. L. Grady, Jr.,(25) can be compared with some of the results of the present study. He recorded values for specific growth rates at 500 mg/l substrate concentration for several amino acids. He found low growth rates with alanine and values for μ of 0.027 and 0.128 hr^{-1} compared with 0.408 and 0.183 hr^{-1} at 600 mg/l obtained in this study. For glutamic acid he found μ values of 0.347 and 0.433 hr^{-1}, whereas in the present study values of 0.730 and 0.508 hr^{-1} at 600 mg/l were observed. For serine he found 0.338 hr^{-1} and values of 0.407 and 0.514 hr^{-1} at 600 mg/l were observed in the present study. For histidine his values were 0.407 and 0.301 hr^{-1} compared to 0.462 and 0.604 hr^{-1} at 600 mg/l for this study. For cysteine he found values of 0.301 and 0.277 hr^{-1} and a value of 0.201 hr^{-1} at 600 mg/l was observed for the present work. Finally, he found μ values of 0.289 and 0.154 hr^{-1} for phenylalanine and in the present study μ values were 0.308 and 0.301 hr^{-1} at 600 mg/l. The compounds that showed low growth rate in this study were also low in his work.

In general, the results from the amino acids experi-

ments supported the Monod relationship although growth on alanine, phenylalanine, and cysteine was not well enough defined to permit a comparison.

The last group of compounds was the fatty acids. Again the cells didn't seem to be able to utilize the substrate very efficiently. The growth rates observed during the first runs for both acetic acid and propionic acid were grouped somewhat closely around the same value regardless of substrate concentration. The values for the second runs were a little more spread out in both cases. The first and second experiments were conducted within three days of each other; it is possible that prolonged acclimation could have permitted selection of cells more capable of higher yields. Neither acid gave a complete μ vs S curve, but there was even less evidence for the linear relationship.

The growth rate studies employing concentrated municipal sewage were particularly interesting since the waste water contains many carbon sources and it was important to determine the relationship between μ and S for the naturally occurring complicated substrate. In the present study flash evaporation was finally used to concentrate the sewage, although it is felt that further experimentation with the freeze drying technique would ultimately prove a more worthy procedure which might retard loss of volatile compounds.

From figures 25 and 26 it is interesting to note that

approximately 50% of the COD in the primary effluent was soluble. The ratio was approximately constant for the first sample concentrated (see figure 25) but for the second sample (figure 26) the COD of the filtered concentrate was somewhat less than 50% of the total COD of the concentrate as the concentration ratio was increased. Less COD was lost when evaporation was conducted at the lower temperatures (compare figures 25 and 26-unfiltered theoretical vs observed). But a higher proportion of the COD which was retained in the concentration flask was lost upon filtering (almost 2/3 of the remaining COD was lost by filtering). The loss of COD due to the concentrating procedure provide an insight into the volatile material present since the evaporation was conducted under vacuum. The condensate was analysed for COD and it never contained any more than about 30 mg/l, therefore, the volatiles were removed by the vacuum. A known concentration of glucose solution was concentrated in the same apparatus; the glucose was retained completely in the concentrate flask. Also a known concentration of acetic acid solution was found to retain 30% in the concentrate flask and 70% in the condensate flask (26). Thus the volatile material in the sewage exhibited a higher degree of volatility than that exhibited by acetic acid under like operational conditions. No attempt was made to determine the nature of the volatile materials, but it can be concluded that the lower temperature of the second

run retarded escape of these compounds. Concerning the unsuccessful attempt to employ freeze drying principles, it is felt that the difficulty was due to the rather small diameter of the vacuum tube in relation to the large volume of the freeze chamber, i.e., the chamber could not be evacuated rapidly enough to prevent melting of the thin layer of frozen sewage on the wall of the freezing flask. It was later discovered that Painter and Viney (27) had used freeze drying on domestic sewage. They concentrated 20 liter batches of composite filtrate to powder. They reported that the work was done by the Microbiological Research Establishment at Porton and by the Torry Laboratory of the Department of Scientific and Industrial Research. Further investigation of the procedure should prove useful in research on municipal sewages, or for that matter on any dilute waste water system.

The concentrated sewage and the filtered concentrate had a pH of 8.6, was dark yellow in color, and had the characteristic odor of sewage. No salts or buffer was added to the substrate. The cells grew on it quickly and only a three day acclimation period was used prior to the first experiment and a five day period for the second experiment. The experiments were conducted in the same manner, but the seed cells were different. Two different batches of concentrated sewage were used, the units were started with different sewage seeds, and the values from the two experiments were very much alike. The values for

μ_m of 0.40 to 0.49 hr^{-1} are very close to those found by Gaudy, Ramanathan, and Rao (1) and by Ramanathan and Gaudy (2) for glucose substrate. The k_s values of 40 to 60 mg/l were a little lower than those other workers observed.

CHAPTER VI

CONCLUSIONS

The results of this study support the following conclusions:

1. In general the experimental results provide evidence for applicability of a single phase hyperbolic relationship between μ and S in preference to a linear two phase relationship for heterogeneous populations of sewage origin.

2. A change in microbial predominance as evidenced by a change in color or types of mixed liquor cell suspension, can change the maximum growth rate considerably.

3. The heterogeneous populations acclimate to a variety of carbon sources. The amino acids tested, in general, could be utilized as well as the carbohydrates. The simple fatty acids seemed to support less growth than the carbohydrates or amino acids.

4. The results of the study on concentrated sewage provided excellent evidence in the support of the single phase hyperbolic relationship between μ and S. The values of μ_m and k_s determined for sewage agreed with those

found in previous studies using glucose which served as a basis for recommended values for carbohydrate wastes. It is noted that a considerable amount of COD was volatilized and subsequently lost during the flash evaporation of municipal sewage. Also only the soluble nonvolatile fraction of the sewage was used as substrate.

CHAPTER VII

SUGGESTIONS FOR FUTURE WORK

1. The study could be extended by including more compounds and combinations of several substrates. This would provide a broader analysis of the many compounds found in industrial wastes.

2. A more detailed study on municipal sewage should be conducted. Samples could be collected at different times of the year and from other cities. This would provide a greater insight into the microbial responses to the various conditions of a treatment plant.

3. The construction and use of the freeze drying technique for concentrating large volumes of sewage should be studied. The resulting residue should contain all of the sewage constituents and provide an excellent media for the analysis of municipal sewage.

PART II

THE EFFECT OF SODIUM AND CHLORIDE IONS ON GROWTH, SUBSTRATE REMOVAL, AND CELL YIELD

CHAPTER I

INTRODUCTION

The reuse of water is becoming more important and more complex every day. After the water has served its purpose, it is discharged as wastewater into a river. If there were no treatments to remove the objectional materials from the water, the river would soon become polluted, all higher forms of aquatic life would die and the river would become an eye sore and a health hazard. Most municipalities are now treating a combined waste consisting of the domestic and industrial wastes. The addition of industrial wastes can complicate the problem of providing efficient treatment of the waste. There are as many different problems in combined waste treatment as there are types of industrial wastes. One constituent found in many industrial wastes is sodium chloride. Industries may employ saline water for cooling, some may use brine for pickling, and still others may use fresh water to wash salts out of the process. In order to design a biological treatment plant, the effect of salinity on the microbial populations and on the substrate removal effi-

ciency of the system must be determined.

Several investigators have reported on the effects of salt on the efficiency of trickling filters and activated sludge processes. Kincannon and Gaudy reported on the effects of various concentrations of sodium chloride on activated sludge for both long term and shock exposures (28) (29) (30). These workers observed an increase in cell yield at low concentrations of salt and a decrease in substrate removal efficiency at high concentrations.

The purposes of the present study were to determine if the presence of an excess amount of sodium ion had any effect on the cell yield, and to determine the effect of various concentrations of NaCl on sludge yield values obtained under batch growth conditions.

It is believed that the results of this study will contribute toward better understanding of the response of activated sludge processes to various salt concentrations found in waste waters.

CHAPTER II

LITERATURE REVIEW

A considerable amount of research has been conducted in the microbiological field on the effects of sodium chloride on bacteria. In general it has been found that the growth of most bacteria is stimulated at sodium chloride concentrations in the range of 0.005M to 0.50M; at higher values growth is retarded (31) (32) (33). Some investigators have reported on the adaptation of bacteria to sodium chloride with increasing tolerance levels being observed (34) (35).

In the pollution control field, investigators have reported on the temporary reduction in treatment efficiency caused by various sodium chloride concentrations, and the time required for recovery of the biological treatment process (36) (37).

Kincannon and Gaudy (38) found that high salt concentrations could cause a severe decrease in specific substrate removal rate in batch grown activated sludges. They also found that long-term exposure to high salt concentrations caused a significant change in the ratio of respiration to synthesis.

Later Kincannon and Gaudy (28), working with batch activated sludge, found a noticeable decrease in specific substrate removal rate when fresh water sludges were shock dosed with 30,000 mg/l NaCl. A more severe impairment was noticed with doses of 45,000 mg/l. When the cells were acclimated to high salt concentrations and placed in fresh water, they lysed. When sludges grown at 45,000 mg/l NaCl were subjected to fresher water under conditions which prevented the osmotic shock, lysis was not noted. This result was taken as an indication that during acclimation to high salts species tolerant to salts came into predominance.

Kincannon, Gaudy and Gaudy (29) observed that when lysis occurred, the system response was characterized by diphasic growth, the first stage being due to glucose metabolism and the second stage to the metabolism of the released cellular constituents. A new enzyme system was needed for the metabolism of the lysate.

Much work has also been done on the mechanism of salt action on the bacteria. Nunheimer and Fabian (39) felt that the effect of a salt was a function of the action of both ions as well as the change in osmotic pressure due to the salt. Fabian and Winslow (40) shared this view.

Clark and MacLeod (41) studied the effects of ions on glycolysis using a cell-free extract of Lactobacillus arabinosus. It was found that inhibition of glycolysis by Na^+ was manifested primarily by an increase in the lag phase preceding the initiation of glycolysis and that,

once established, glycolysis proceeded at a rate which was almost unaffected by the presence of Na^+.

Kincannon and Gaudy (30) studied the effect of NaCl on the yield of biological solids and the ability of continuously cultured heterogeneous microbial populations to remove substrate at different salt concentrations in the inflowing synthetic waste. They found the system could not maintain a high substrate removal efficiency during the period of increasing salt concentration from zero to a level of 30,000 mg/l. After an acclimation period the system regained its former efficiency. When the salt was diluted out of the system, a significant rise in cell yield was found as the salt level passed through the range 8,000 - 10,000 mg/l. It was found that steady operation at a salt level of 8000 mg/l sustained the cell yield at a high level.

CHAPTER III

MATERIALS AND METHODS

A. Experimental Protocol

1. Effect of Sodium Ion in the Growth Medium

Two batch activated sludge units (1.5 liter aeration volume) were developed from an initial sewage seed taken from the effluent of the primary clarifier of the Stillwater, Oklahoma, municipal sewage treatment plant. The synthetic waste was identical for the two units except for the buffers. One reactor, hereafter referred to as unit K, was the control and contained a buffer mixture of monobasic and dibasic potassium phosphate; the other reactor, unit Na, contained a buffer mixture of monobasic sodium phosphate and dibasic potassium phosphate. The composition of the growth medium was as follows: glucose, 1000 mg/l; $(NH_4)_2SO_4$, 500 mg/l; $MgSO_4.7H_2O$, 100 mg/l; $FeCl_3.6H_2O$, 0.5 mg/l; $MnSO_4.H_2O$, 10 mg/l; $CaCl_2$, 7.5 mg/l; potassium unit buffer, K_2HPO_4, 1070 mg/l; KH_2PO_4, 527mg/l; sodium unit buffer, K_2HPO_4, 1070 mg/l; NaH_2PO_4, 311 mg/l; tap water, 100 ml/l. Air was supplied to both units at a rate of 1.5 liters of air/min/liter of solution.

Each unit was operated in accordance with the following procedure. Daily, 500 ml of the mixed liquor were wasted and the remaining liquor was allowed to settle for one hour. Then 500 ml of the supernatant were wasted and the units were brought to the 1.5 liter volume with synthetic waste and distilled water. Experiments on these systems were made after they had attained a solids balance.

Twelve experiments were conducted and these are summarized briefly in Table III. The first four experiments were conducted to determine if the presence of Na^+ had any effect on the cell yield and other system parameters. In addition to cell yield, specific growth rate, μ, specific substrate removal rate, SRR, and the oxygen uptake were measured. At the beginning of each experiment (zero time) the synthetic waste and sludge were fed to each unit. Two 40 ml samples were taken from each unit, one sample was placed in a Warburg flask for measurement of oxygen uptake, and one was used for measurement of biological solids and COD concentrations. The latter sample was centrifuged (when necessary) in a Servall centrifuge and then filtered through a Millipore filter. The filtrate was used for the COD determination and the residue was used for the solids determination. This procedure was repeated at various time intervals for the measurement of substrate removal and biological solids accumulation.

TABLE III

DESCRIPTION OF NaCl EXPERIMENTS

Exp. No.	Type of Experiment	Remarks
1	Comparison of potassium and sodium buffer	K unit - K = 0.39 gm/l Na unit - K = 0.24 gm/l Na = 0.60 gm/l
2	Same as above	Same as above
3	Same as above	Same as above
4	Same as above	Same as above
5	Same as above	K and Na units both with K unit cells
6	Same as above	K and Na units both with Na unit cells
7	Fresh water cells shocked with NaCl	Concentrations used 333, 667, 1333 mg/l
8	Same as above	Concentrations used 2000, 2667, 3333 mg/l
9	Same as above	Concentrations used 4000, 4667, 5333 mg/l
10	Same as above	Concentrations used 6000, 6667 mg/l
11	Same as above	Concentrations used 7000, 8000, 9000, 10,000 mg/l
12	Cells acclimated to 30,000 mg/l NaCl used for salt dilute-out	Concentrations used 30,000, 25,000, 20,000, 15,000, 10,000 mg/l

Experiments five and six were conducted differently. In experiment number five, two units were started with a seed from the standard unit K, one with potassium buffer and one with sodium buffer. In experiment number six, two units were started with the seed from the standard unit Na, one with potassium and one with sodium buffer. The sampling procedure remained the same as before.

2. Various NaCl Concentrations

Unit Na was discarded after experiment number six and in the remaining experiments cells from the standard unit K were employed. Experiments seven through eleven were made to determine if various NaCl concentrations would increase the sludge yield. In all of these experiments, a fresh water control unit was run as well as a series of similar reactors which received varying concentrations of NaCl. The range of NaCl concentrations investigated was 0 - 10,000 mg/l. The sampling techniques were the same as previously described.

From the results of the above experiments it was decided to acclimate a system to 30,000 mg/l NaCl. The system was developed from a combination of sewage seed and "old" cells from standard unit K. The NaCl was increased in the unit to 30,000 mg/l over a period of a few days. After a period of acclimation to the higher concentration of NaCl the cells were employed in a growth experiment at various NaCl concentrations below 30,000

mg/l. The NaCl concentrations employed were as follows: 30,000 mg/l, 25,000 mg/l, 20,000 mg/l, 15,000 mg/l, and 10,000 mg/l NaCl. The fresh media was prepared for each experimental unit and contained the correct salt concentration. The sampling procedure for this experiment was the same as previously described.

B. Analyses

1. Biological Solids

Biological solids concentrations were determined by the membrane filter technique (42). Aluminum dishes (weighing approximately 0.2gm) were used to hold the Millipore filters, (HA, 0.45 μ).

2. Substrate Removal

The Chemical Oxygen Demand procedure was followed as outlined in Standard Methods (42) for a 40 ml and 20 ml sample size. Mercuric Sulfate ($HgSO_4$) was added to prohibit the excess chlorides from interfering with the dichromate reaction. A Hack spoon held approximately 0.4 gm of $HgSO_4$ which would complex the chloride ion contained in approximately 3300 mg/l NaCl. This procedure was not necessary for the first seven experiments.

3. Oxygen Uptake - Warburg Unit

Flasks were prepared by placing one milliliter of 20% KOH in the center well to absorb CO_2. Forty ml of reaction liquor was added to the sample flasks, and 40 ml

of distilled water was used in the blank (barometer) flask. The flasks were then attached to their respective manometers, and placed in the constant temperature water bath at 25°C. After equilibration the manometers were set and closed; readings were taken at various time intervals. After correcting each reading for the barometer flask deflection the accumulated oxygen uptake was calculated using previously determined flask constants.

CHAPTER IV

RESULTS

A. Effects of Sodium Concentration in the Growth Medium

The first experiments of this study were made primarily to determine if an increased amount of sodium in the synthetic waste would have any effect on the cell yield. A "regular" (routinely employed) potassium phosphate buffered system with only trace amounts of sodium and a unit buffered with a mixture of sodium and potassium (0.60 gm/l Na) were maintained throughout the six experiments. Figure 33 shows the performance of these systems with respect to biological solids and COD concentrations during the experimental period. The arrows indicate the time of running experiments one through six. It is seen that there were no drastic differences in the behavior of these systems with regard to biological solids level and COD removal efficiency.

In early March a change in predominance took place. Both units developed "clumped" cells. The settleable solids were finally dispersed by short periods of mixing in a Waring blender (March 9). Unit Na required two additional blendings, but finally both systems grew in a

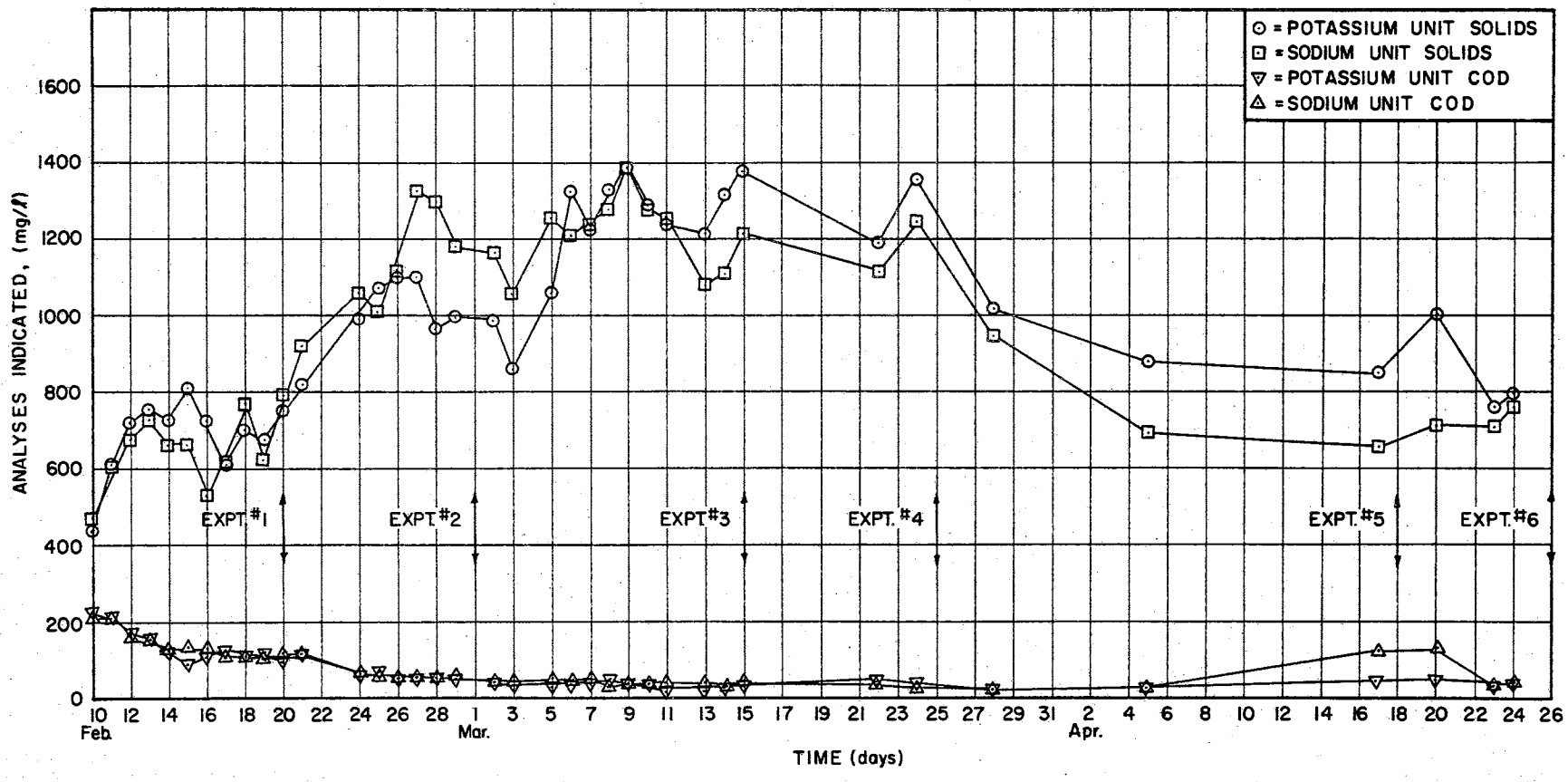

Figure 33. System Performance in Control Units Sampled 24 hours after Feeding. One Unit Contained Potassium Buffer and One Sodium-potassium Buffer

more dispersed manner. On March 24, the systems began to manifest different appearances, in the potassium unit the mixed liquor became much darker than in unit Na. On April 13, both systems exhibited a greenish-brown color and there was less tendency for the cells to flock during the settling period. On April 24, the unit Na exhibited a darker color than the unit K. Also, the balance of flock in the sodium unit amounted to nearly one liter after settling while the potassium unit did not settle at all. Both units had the same appearance (i. e., color) when experiment five was run, while the appearance of the units was the same as that on April 24 when experiment six was run.

The results for an experiment typical of the first four runs (experiment 2) are shown in figures 34, 35, 36, and 37. From figure 34 it is seen that there were no readily apparent differences in sludge accumulations and COD removal for these systems. In figure 35 the biological solids concentration and the concentration of COD removal at each successive sampling time are shown in semi log plots. From the slope of these lines the specific growth rates and substrate removal rate constants were calculated. In figure 36, the substrate removed is plotted versus the biological solids produced in a given time period. The slope of the line is the value for cell yield. Oxygen uptake during experiment two is plotted in figure 37. Triplicate flasks were employed for each system.

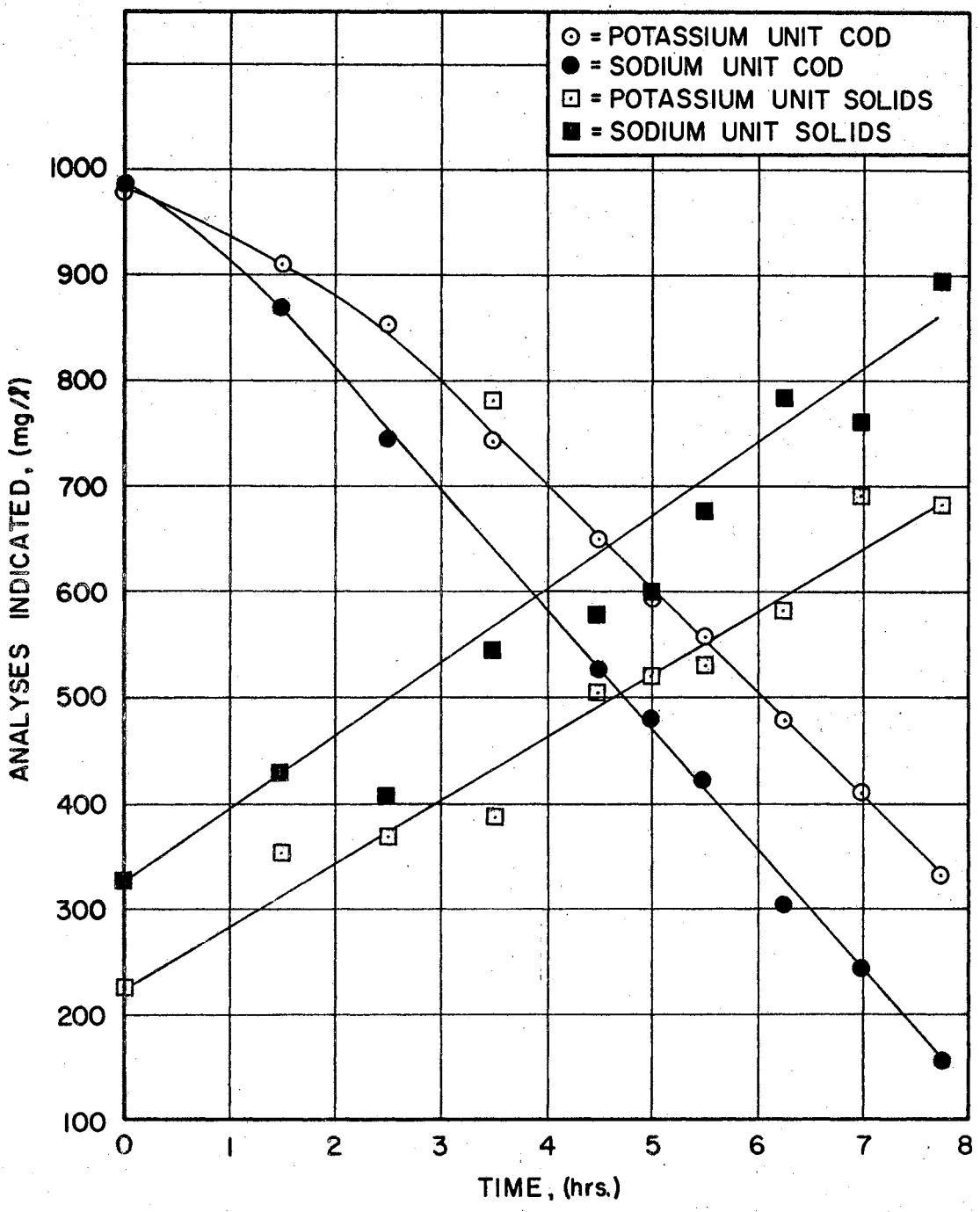

Figure 34. System Performance in Units for Experiment 2

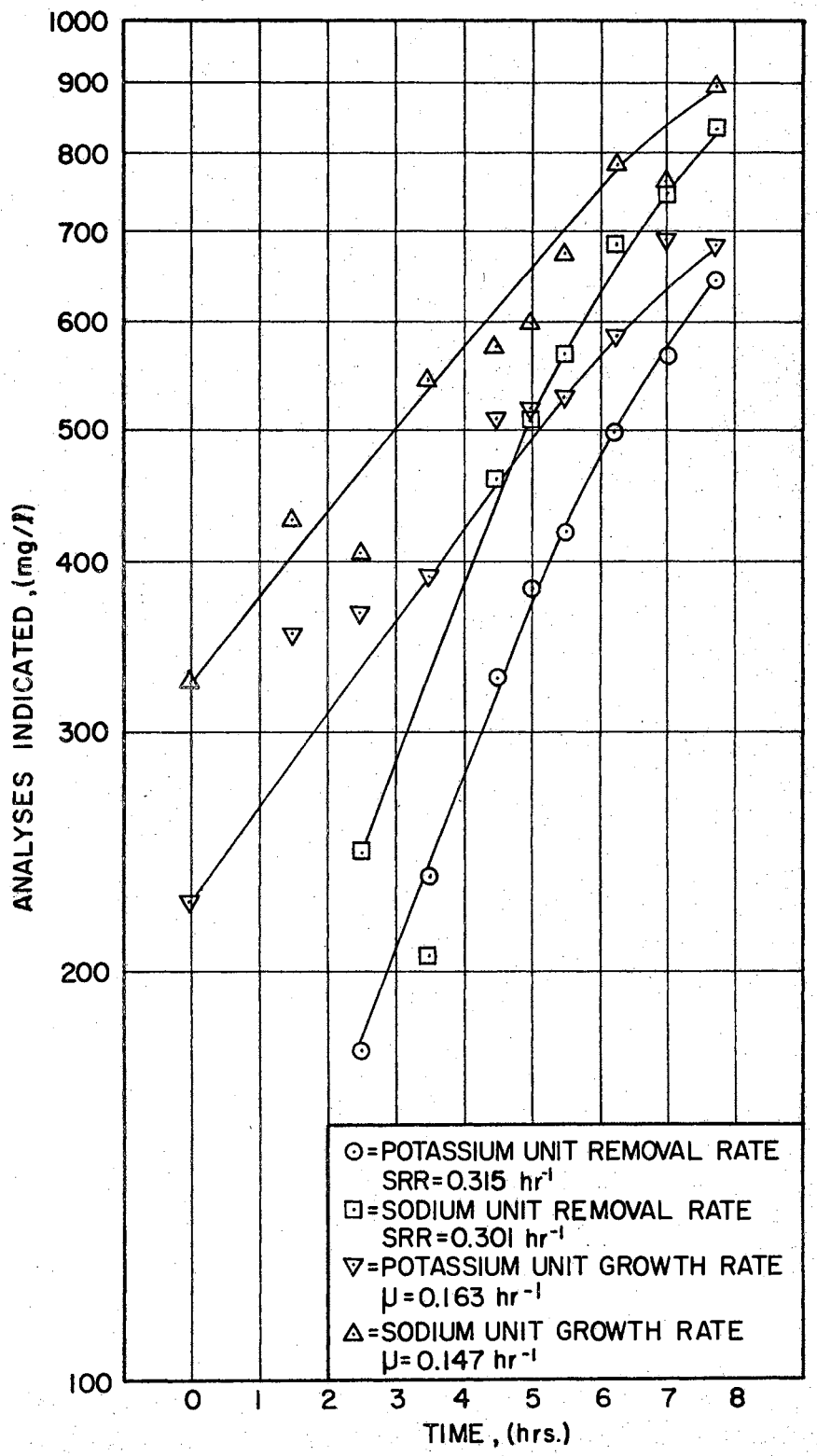

Figure 35. Substrate Removal and Growth Curves for Potassium and Sodium Units

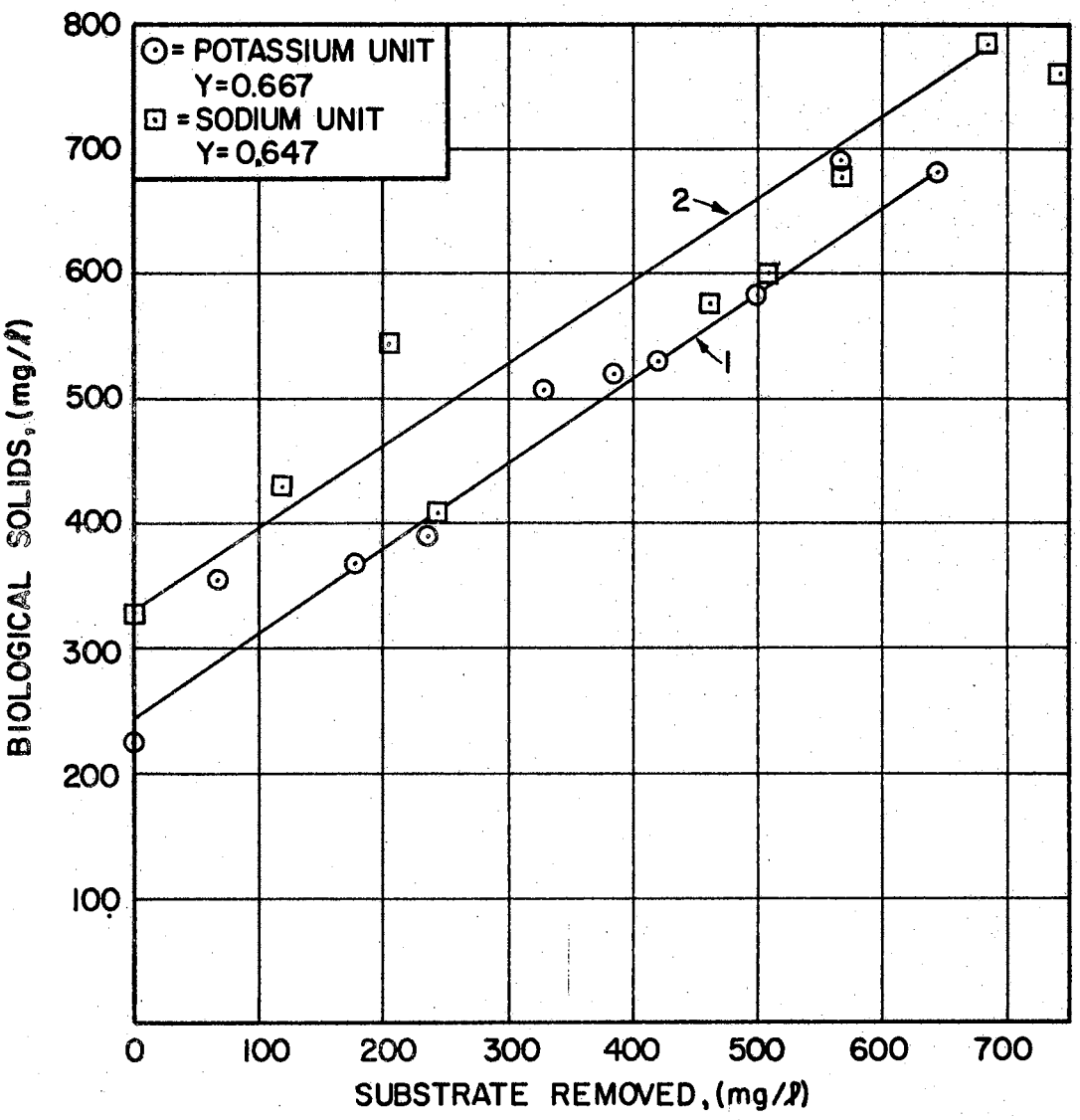

Figure 36. Relationship between Biological Solids and Substrate Removed Used to Calculated the Yields

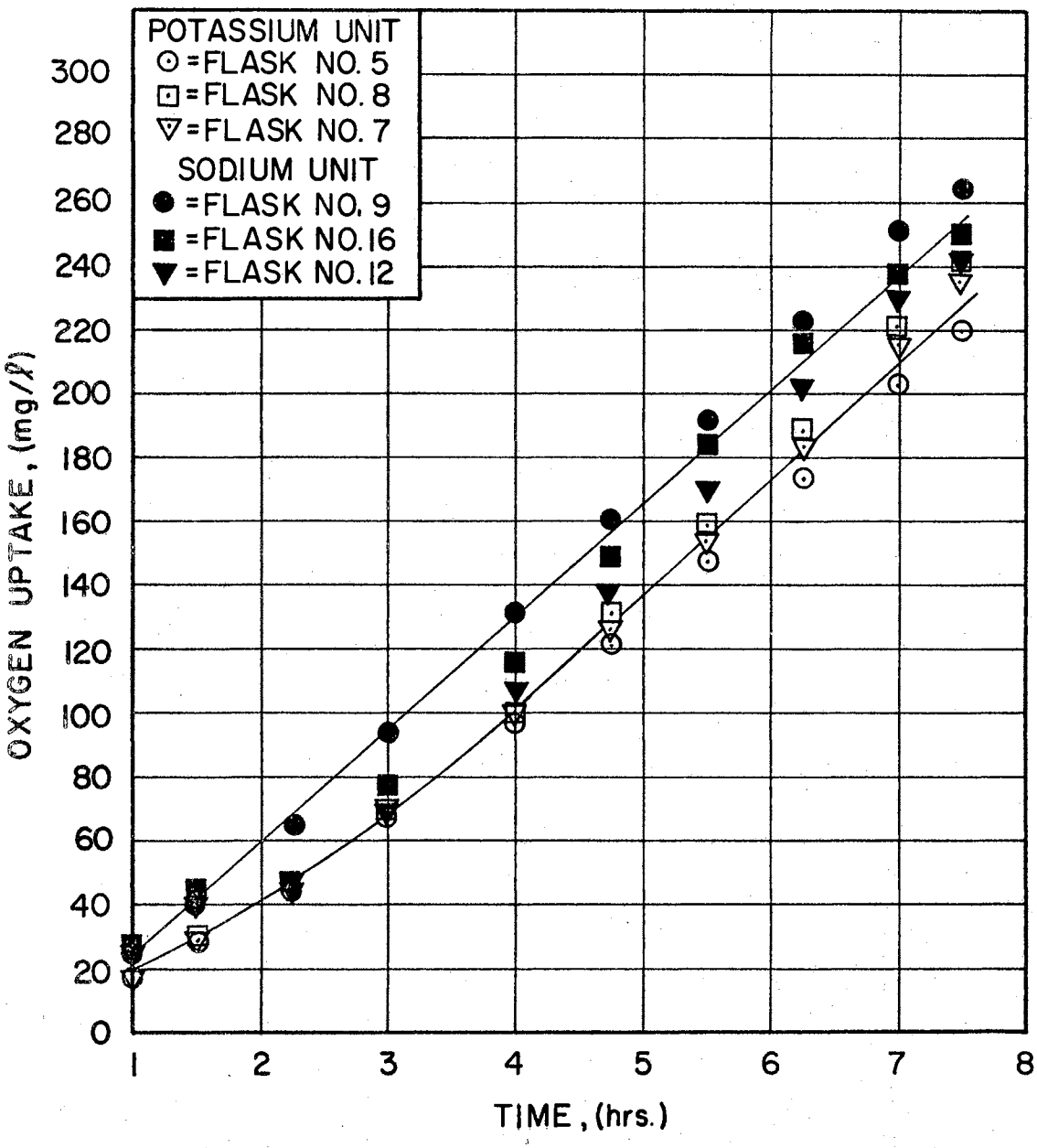

Figure 37. Oxygen Uptake Plots for Potassium and Sodium Units for Experiment 2

The values of the various parameters examined for all four experiments are summarized in Table IV. It is seen, thus, there would not appear to be any large differences in the values of these growth parameters attributed to the buffer compositions.

In experiment five two batch units were prepared in the same manner as the previous ones. They were seeded with cells from the standard potassium system (unit K). One reactor contained the standard synthetic waste with potassium buffer, and the other contained the sodium buffer. Figure 38 shows the COD and solids concentration in both units, as the experiments proceeded; there was essentially no difference in the response of these systems. Separate determinations of the substrate removal rate constants showed that they were 0.189 hr^{-1} for the potassium system and 0.182 hr^{-1} for the sodium system. The specific growth rate was 0.099 hr^{-1} for both systems. The yield values were 0.63 for the potassium system and 0.58 for the sodium system.

Experiment six was run in a manner similar to experiment five except that sludge from the standard sodium system was employed. In figure 39 it is seen that the values for either system fit a single curve. The values for substrate removal rate constant and growth rate constant obtained for the potassium buffer system and the sodium buffer system were; 0.177 hrs^{-1} for both, 0.100 hr^{-1} and 0.098 hr^{-1}, and the yield constants 0.62 and

TABLE IV

SUMMARY OF RESULTS OF EXPERIMENTS 1-4

Exp. No.	Duration (hrs)	Initial COD (mg/l)		Final COD (mg/l)		Initial Solids (mg/l)		Final Solids (mg/l)		Oxygen Utilization (mg/l)		SRR (hr^{-1})		μ (hr^{-1})		Y	
		K	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K	Na
1	7 3/4	980	960	505	340	195	185	470	565	260	295	0.247	0.266	0.117	0.161	0.58	0.75
2	7 3/4	980	990	335	155	225	330	685	895	230	255	0.315	0.301	0.163	0.147	0.67	0.65
3	7 3/4	940	945	520	590	220	215	490	440	145	120	0.308	0.252	0.165	0.218	0.63	0.64
4	11 1/2	985	1025	20	230	100	90	735	600	155	135	0.138	0.120	0.099	0.101	0.72	0.67

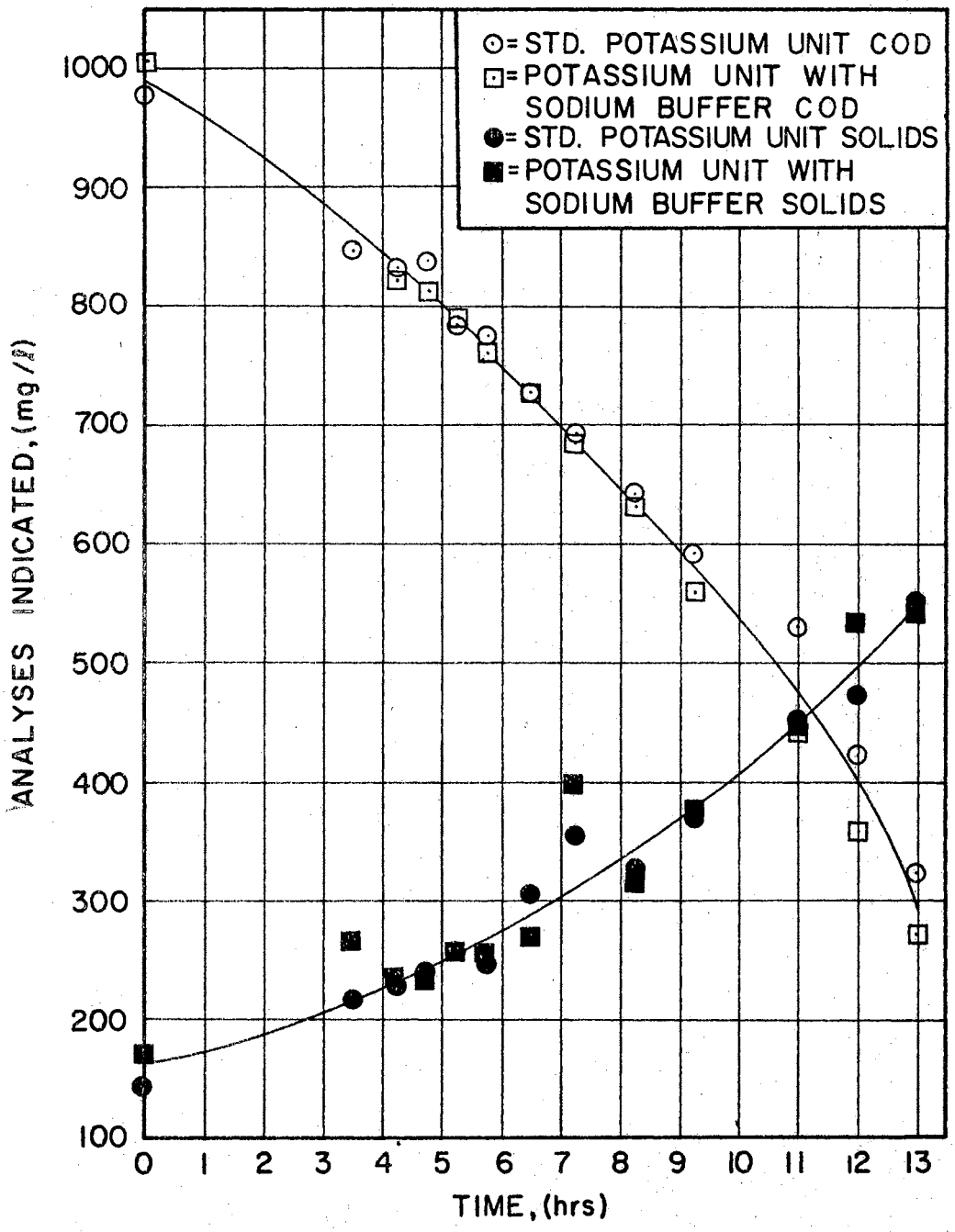

Figure 38. System Response in Units for Experiment 5. Cells from Potassium Unit Used as Seed for Both Units.

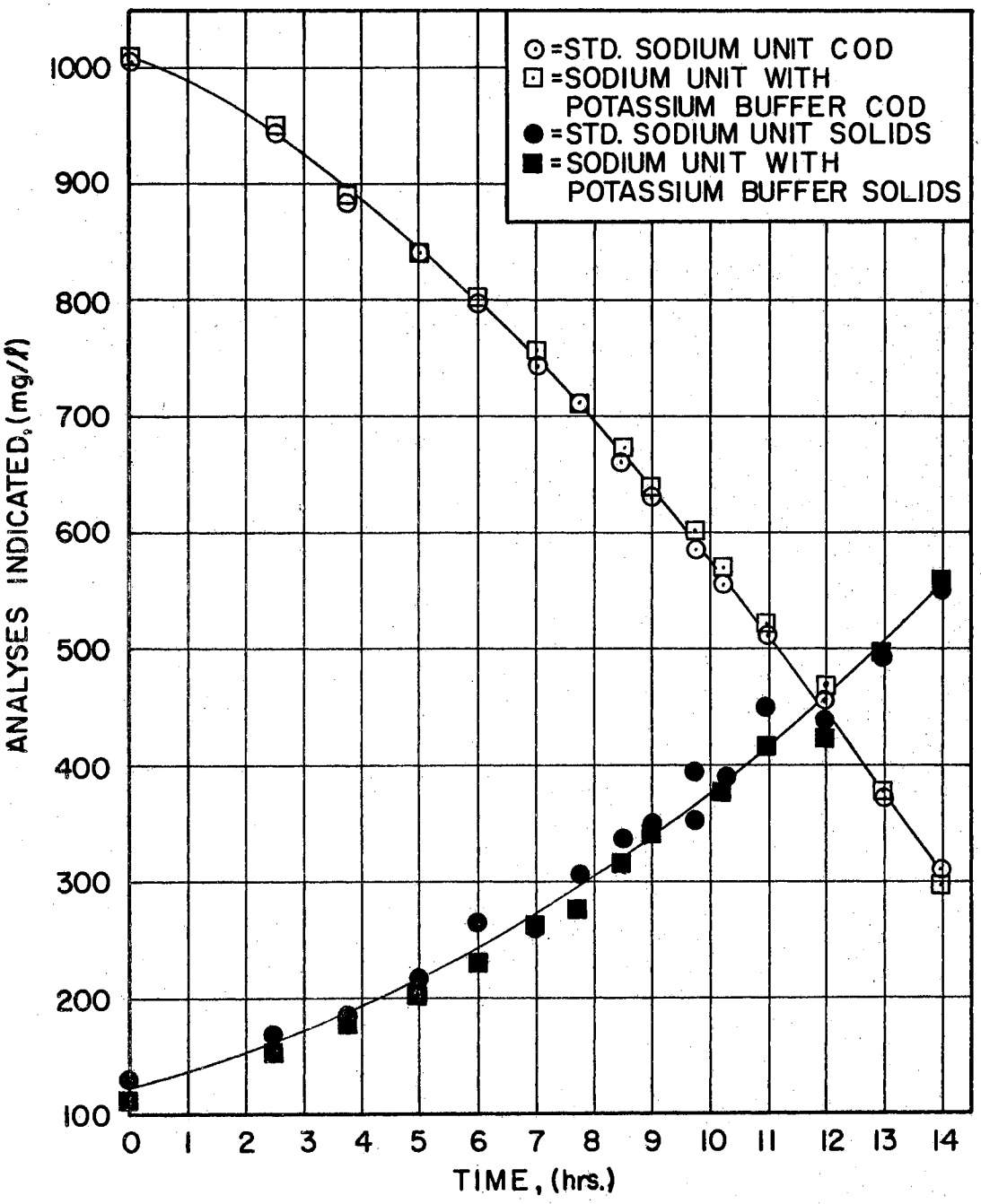

Figure 39. System Response in Units for Experiment 6. Cells from Sodium Unit Used as Seed for Both Units.

0.60. These results like those from experiment five indicate that the salts composition employed in the buffer did not affect the values of the growth parameters.

B. The Effect of NaCl on Cell Yield

Experiments one through six provided evidence that the addition of sodium in the buffer had little or no effect on the system. However, Kincannon had observed an increase in cell yield at certain levels of NaCl. Therefore, investigational effort was focused on the effect of chloride ion. Experiments seven through eleven were designed to study the response when cells grown in "fresh" water medium were shocked with a series of NaCl concentrations up to 10,000 mg/l. The first of these (experiment 7) was selected as an example for detailed presentation. A fresh water standard (control unit) was run on each experiment. The sampling procedure and analytical techniques were the same as for the previous experiments, except that $HgSO_4$ was added to the COD flasks to complex chlorides.

In figure 40 it is seen that the COD and solids concentration data for all three salt concentrations (333, 667, and 1333 mg/l) could be rather closely fitted with a single curve. Growth rate constants and substrate removal rate constants were calculated from the semi log plots shown in figure 41.

In figure 42 it is seen that the yield values were

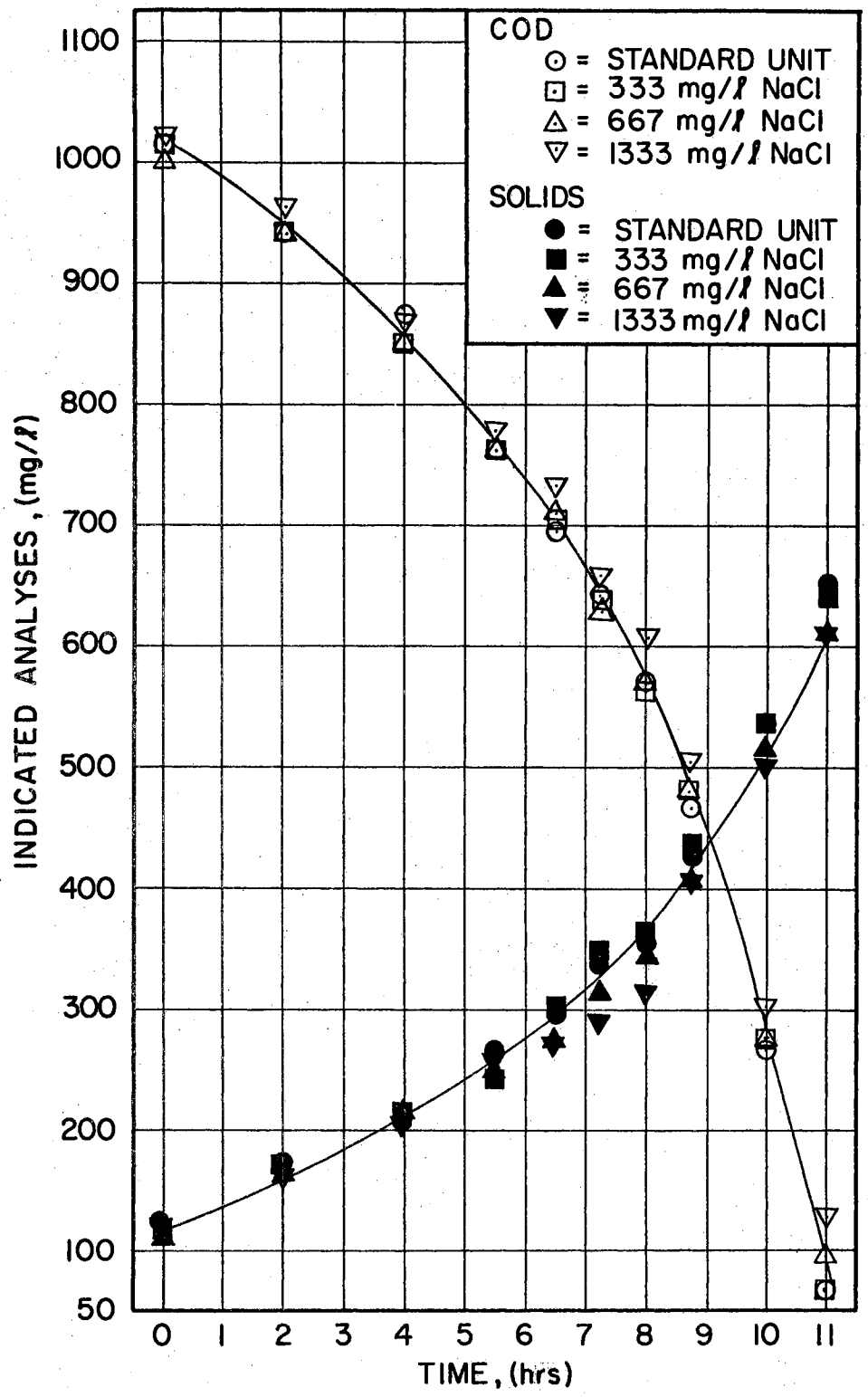

Figure 40. System Response in Fresh Water Control Unit and Units Shocked with 333, 667, and 1333 mg/l NaCl

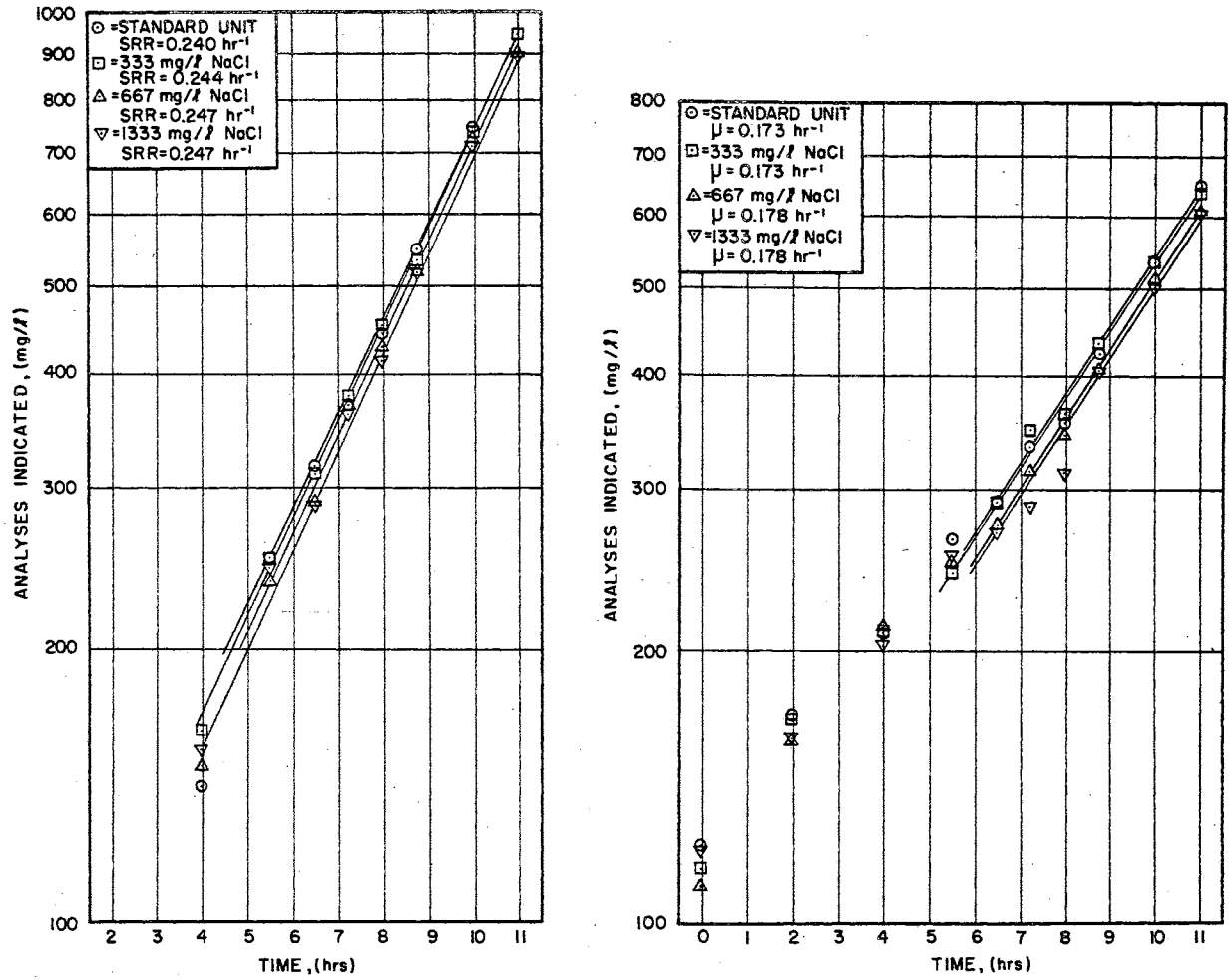

Figure 41. Substrate Removal Curves for Fresh Water Unit and NaCl Shocked Units. Growth Curves for Same Units

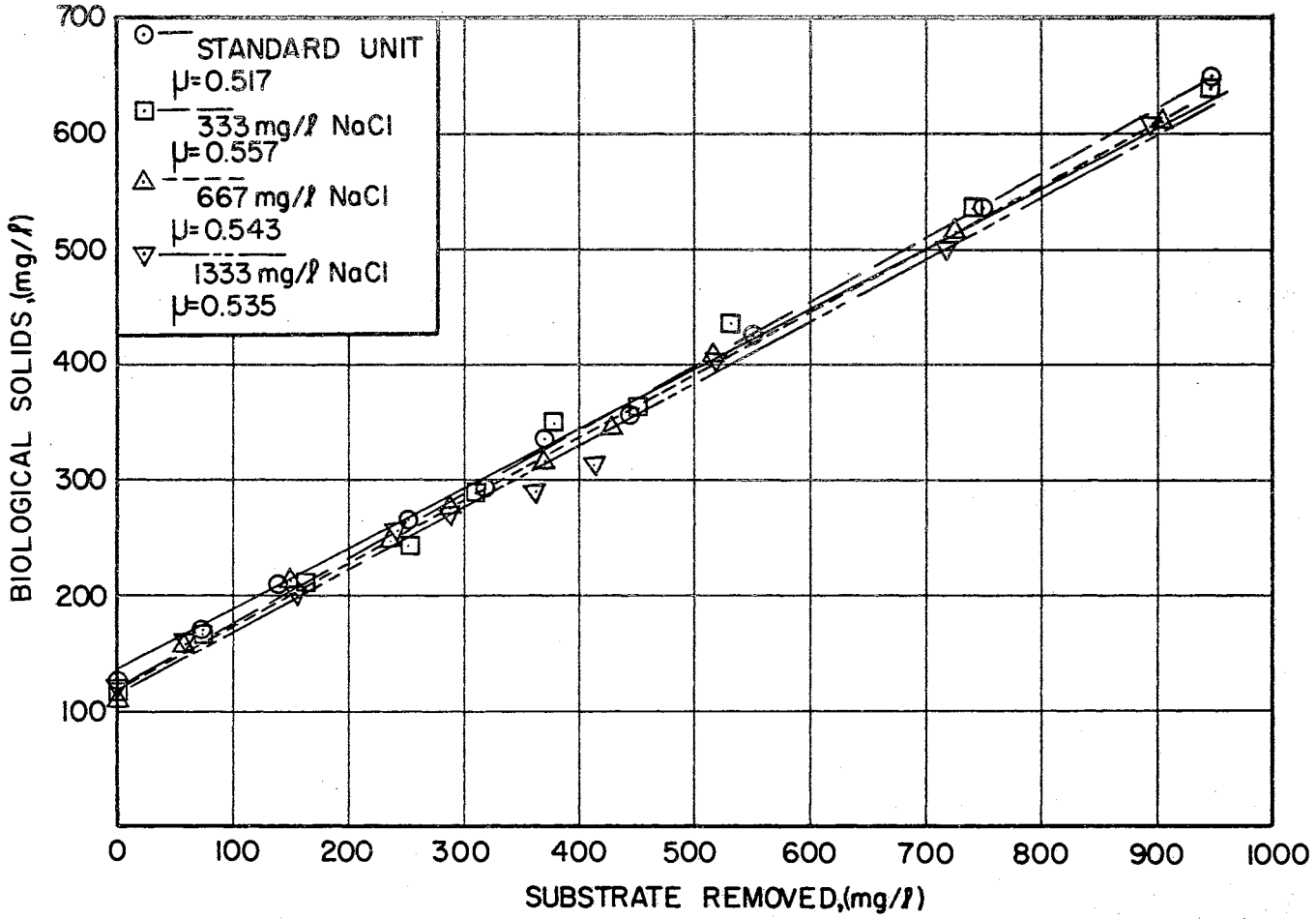

Figure 42. Effect on Cell Yield by NaCl compared to Fresh Water Control Unit. Yields Calculated by Least Squares

very similar for experiment seven. The slope of each line (the yield constant) was determined by the least squares method. The equations used were as follows:

$b = \Sigma xy / \Sigma x^2$ where

$\Sigma xy = \Sigma XiYi - \Sigma Xi \Sigma Yi / n$ where

Xi = substrate removed

Yi = growth

$x = (Xi - \bar{x})$

$y = (Yi - \bar{y})$

$\bar{x}$ = ave. of Xi

$\bar{y}$ = ave. of Yi

The y intercept $a = \bar{y} - b\bar{x}$.

Table V shows the yield values and the significant parameters for experiments seven through eleven. The largest difference in yield values between the NaCl and fresh water systems was observed in experiment eleven at the 8000 mg/l NaCl level (0.59 vs 0.71). Since for each experiment there was also a fresh water standard, and because yield values for the standard varied, the results were compared on the basis of the "yield ratio." This value was calculated by dividing the yield for each NaCl concentration by the yield for the fresh water standard in that experiment. These values are given in Table V and they range from 1.00 to 1.20. Thus while NaCl concentrations up to 10,000 mg/l did not cause drastic changes in the yield coefficient they did cause a noticeable increase. Kincannon observed very high cell yield in con-

TABLE V

SUMMARY OF RESULTS FOR NaCl EXPERIMENTS

Exp. No.	NaCl Conc. mg/l	Na^+ mg/l	Cl^- mg/l	Initial COD mg/l	SRR hr^{-1}	Initial Solids mg/l	μ hr^{-1}	Yield	Yield Ratio
7	fresh	0	0	1010	0.240	123	0.173	0.52	-
7	333	131	202	1010	0.244	115	0.173	0.56	1.077
7	667	263	404	1000	0.247	110	0.178	0.54	1.050
7	1333	525	808	1020	0.247	120	0.178	0.54	1.035
8	fresh	0	0	940	0.210	113	0.126	0.52	-
8	2000	790	1210	965	0.210	108	0.135	0.58	1.112
8	2667	1053	1614	915	0.213	103	0.148	0.57	1.099
8	3333	1315	2018	965	0.216	103	0.130	0.53	1.017
9	fresh	0	0	1050	0.185	110	0.117	0.57	-
9	4000	1580	2420	1060	0.161	110	0.124	0.58	1.023
9	4667	1843	2824	1040	0.159	103	0.110	0.57	1.004
9	5333	2105	3228	1030	0.169	100	0.129	0.58	1.016
10	fresh	0	0	970	0.187	95	0.143	0.60	-
10	6000	2370	3630	1000	0.181	123	0.146	0.62	1.020
10	6667	2633	4034	990	0.165	95	0.139	0.66	1.086
11	fresh	0	0	945	0.175	93	0.126	0.59	-
11	7000	2765	4235	960	0.161	95	0.130	0.61	1.043
11	8000	3160	4840	950	0.161	93	0.115	0.71	1.210
11	9000	3555	5445	975	0.182	95	0.121	0.61	1.036
11	10000	3950	6050	980	0.161	90	0.121	0.62	1.056

tinuous flow systems during dilute out of NaCl from a previous steady state concentration of 30,000 mg/l. Therefore, in the present study, batch experiments were performed after acclimating the control system to 30,000 mg/l NaCl. The unit was acclimated to 10,000 mg/l NaCl for five days then the salt was increased to 30,000 mg/l and operated at the level of salt for eight days before beginning experimentation. The solids remained completely dispersed and did not settle in a one hour sedimentation time. The course of biological solids accumulation and substrate removal were observed in five systems dosed with the following concentrations of salt: 30,000, 25,000, 20,000, 15,000, and 10,000 mg/l NaCl (experiment 12). Large amounts of slimy material which developed made it difficult to filter the samples and only a few sampling points could be obtained before exhaustion of the substrate. The results are shown in figure 43. The speed of substrate removal and solids growth increased with decreasing NaCl concentration. The cell yield values for the 30,000, 25,000, 20,000, 15,000, and 10,000 mg/l NaCl units were 0.45, 0.60, 0.66, 0.54, and 0.52 respectively. A definite increase in yield is indicated with a decrease in NaCl.

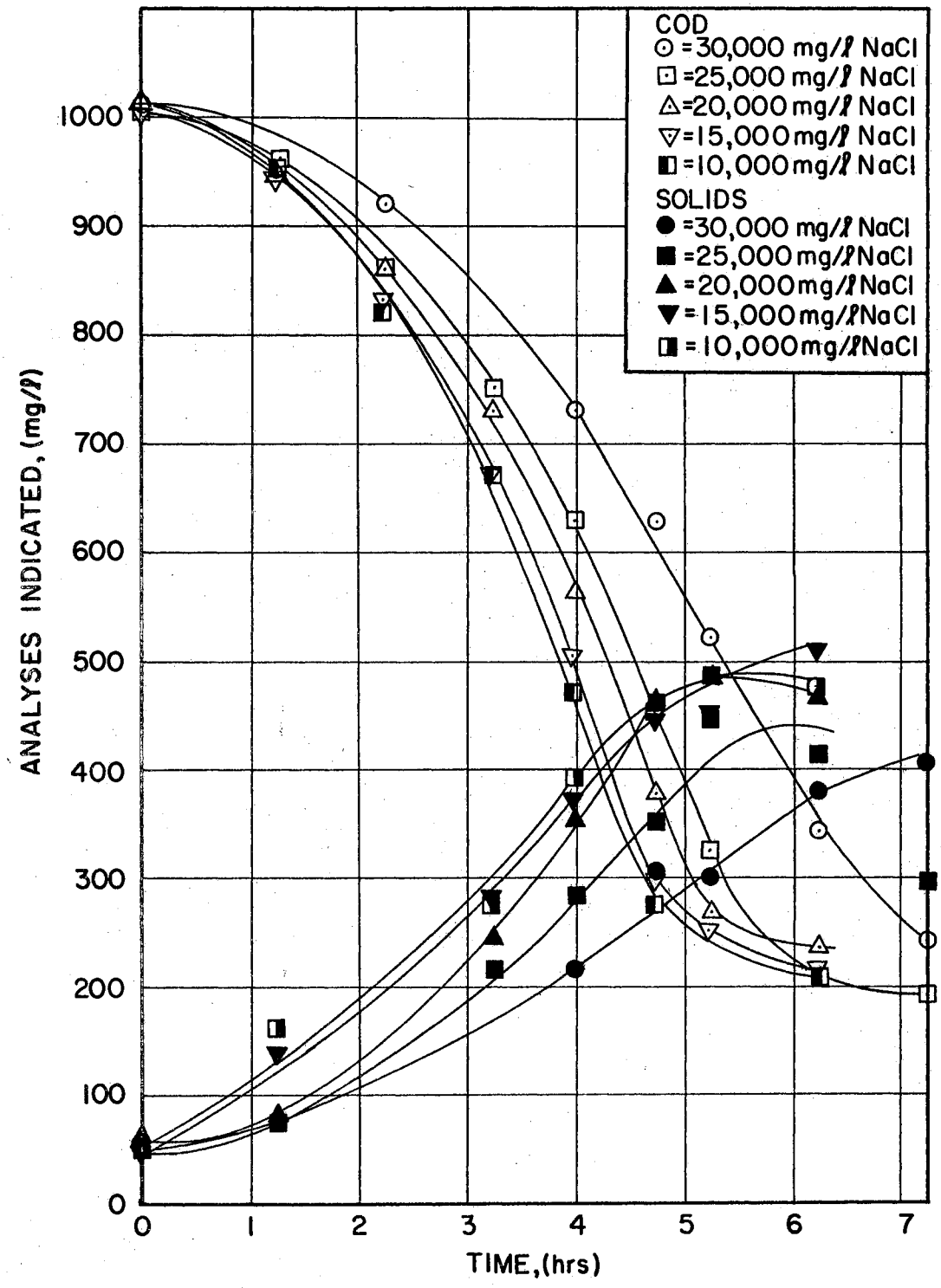

Figure 43. System Response in 30,000 mg/l NaCl Control Unit and NaCl dilute-out Units.

CHAPTER V

DISCUSSION

A. The Effect of Sodium Concentration in the Growth Medium

It is noted that the standard synthetic waste (growth medium) used in this bioengineering laboratory is a minimal salts medium which contains nitrogen and phosphorus in excess as well as various amounts of inorganic salt needed by the bacteria. It is one designed so that the limiting nutrient is the carbon source. However, it may be noted that it contains no sodium salt other than what may be present in the tap water which is routinely added to the medium. It was therefore of interest to determine if the lack of sodium had any gross effect on the growth patterns of the system. Accordingly batch activated sludges with and without added sodium were run for comparative purposes. In the first six experiments the growth patterns for the regular buffer with 0.39 gm/l of K and the sodium buffer with 0.24 gm/l K and 0.60 gm/l Na were compared.

It is interesting to comment on the changes in microbial predominance which occurred during the period

of experimentation. Changes in the color and appearance of the mixed liquor provide some indication of changes in predominance. Both units exhibited a green color during the first week of operation and after 10 days unit Na changed to a brown color. After 20 days both units exhibited a brownish color and the cell existed in large readily settling clumps. After five weeks the potassium unit was darker brown than the sodium unit. In the sixth week heavily flocculated growth developed and the mixed liquor in the potassium unit was dark brown while the sodium unit exhibited a pink hue. After two months, the mixed liquor in both units was a light brown color and both units contained clumped cells. After 2½ months the reaction liquor in the sodium unit exhibited a darker brown color than that of the potassium system. Thus both units underwent variable changes in predominance and neither exhibited any special or unique appearance which might be attributed to the presence or absence of sodium ion.

As the results in Table IV indicate, there was no large differences in the values of the various parameters examined which could be attributed to the presence or absence of sodium ion. From the results of the first four experiments the widest difference in yield values was 0.17 (experiment 1). For the other experiments the difference in cell yield was no larger than 0.05.

B. The Effect of NaCl on Cell Yield

It has been seen that in the present study the presence of sodium ion caused no noticeable effect on the cell yield. However, during experimentation with a completely mixed growth system Kincannon and Gaudy (30) observed an 80% increase in volatile solids concentration when the feed was changed from a fresh water medium to one containing 8000 mg/l NaCl. Also during dilute-out of salt from a unit which had been previously fed 30,000 mg/l NaCl, the volatile solids concentration rose rapidly to twice the previous steady state concentration as the NaCl level passed through a concentration of 7500 mg/l; the solids level dropped sharply as the salt concentration was lowered further. The second part of the present study was undertaken to determine if the phenomenon was of general occurrence and if this effect on cell yield would be discernible under batch growth conditions.

The only change in analytical procedure from that employed by Kincannon and Gaudy was in the COD determination. The $HgSO_4$ procedure in Standard Methods indicates that 0.4 gm of $HgSO_4$ will complex the chloride in 3300 mg/l NaCl, and states that more should be added for high chloride concentration. This was done and there were no unusually high COD titrations; in all studies COD was removed to low levels and it is assumed that all the chlorides were complexed. From Table V it is seen that slightly higher cell yields were obtained when NaCl was

present in the medium and the greatest increase in cell yield occurred at the 8000 mg/l NaCl level. The yield in the presence of this salt concentration was only 21% higher than in the fresh water control.

The results of the yield calculations from experiment twelve indicate that the highest yield occurred at 20,000 mg/l NaCl and that it dropped off somewhat at the lower level of salt concentration. Although there is a definite indication that lowering the NaCl level from 30,000 mg/l to 10,000 mg/l effected a higher cell yield, the absolute values are not considered to be very accurate because the yield was obtained as the slope of the line joining values of biological solids concentration versus COD removed and it may be seen that the definition of the line could have been improved if it had been possible to obtain a larger number of samples during the substrate removal period. However, the results shown in figure 43 leave little doubt that substrate removal and biological solids growth proceeded more rapidly with decreased NaCl concentrations. In general the results of the present study tend to substantiate the results of Kincannon and Gaudy (28) (30). However the increase in cell yield observed at the salt level of 8000 mg/l were not as large as those observed in the previous study.

CHAPTER VI

CONCLUSIONS

From the results of this study the following conclusions can be made:

1. No differences in the values of growth parameters were observed in systems employing the standard K buffer and a combined K - Na buffer.

2. When "old" fresh water cells are shocked with doses of NaCl through 10,000 mg/l, a small but noticeable change in yield occurred.

3. After cells were acclimated to 30,000 mg/l NaCl for one week, the efficiency of substrate removal was as good as it had been before the application of salt. Completely dispersed growth was observed in the salt acclimated system.

4. When the sludges acclimated to 30,000 mg/l NaCl were transferred to lower concentrations of salt, an increase in substrate removal rate and growth rate was noticed.

CHAPTER VII

SUGGESTIONS FOR FUTURE WORK

1. The same type of study could be conducted to include other metal ions or inorganic salts. This would provide a broader knowledge of the effect of various compounds on the microbial growth and substrate removal in a treatment plant.

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