STUDIES OF ACCLIMATED NATURAL MICROBIAL POPULATIONS GROWN ON ACETIC ACID. I. SUBSTRATE REMOVAL AND ENDOGENOUS PHASES. II. THE EFFECT OF SUBSTRATE CONCENTRATION ON EXPONENTIAL GROWTH RATE CONSTANT

By

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CHAPTER I

INTRODUCTION

For the bioenvironmental engineer and scientist, research and development of physical, chemical, and biological processes for prevention and retardation of ecological degradation in the environment, i.e., life support system, are of paramount concern.

Water pollution abatement and maintenance of conditions of high quality in the aqueous environment are often accomplished by treatment and disposal of industrial and municipal wastewaters, using activated sludge, mixed microbial populations which utilize the organic pollutants to produce new cells; the ultimate end products are carbon dioxide and water. The extended aeration activated sludge or total oxidation system is a biological process which provides purification of wastewater containing organic matter capable of exerting a biochemical oxygen demand. The purification is brought about through assimilation and oxidation. Ultimate disposal of the net growth of cells due to the organic matter in the wastewater is brought about by endogenous respiration or aerobic auto-oxidation of the sludge mass. The research investigation herein is concerned mainly with bacterial metabolism and microbial growth kinetics during substrate removal and with the degree of autodigestion and its kinetics in the subsequent endogenous phases of once-fed batch activated sludge populations of sewage origin, fed a

low molecular weight organic compound (potential pollutant) to which the population had been previously acclimated.

In general, growth of natural microbial cultures can be described using three metabolic "constants:" sludge yield (Y) and the physiological growth "constants," maximum exponential growth rate (μ_{max}), and saturation constant or geometrical curve descriptor (K_s). Cell or sludge yield informs the bioengineer as to the fraction of biochemically available carbon source (ΔCOD) of an exogenous substrate that is channelled into cellular mass during the metabolism of bacterial cultures and, consequently, the accumulation of a sludge mass eventually to be disposed of. It is generally assumed that cell yield is constant during logarithmic growth of microorganisms, but operational sludge yield data are usually obtained after the exponential phase, i.e., during either the declining growth or the stationary phases. The investigation herein is in part addressed to determination of the constancy of cell yield during the entire course of autocatalytic growth of microorganisms on a non-carbohydrate, low molecular weight organic compound (acetic acid).

The kinetic growth constants, specific growth rate (μ) and saturation constant (K_s), can be considered as mathematical descriptors for kinetic behavior of growing microbial systems. These "constants" when inserted into the hyperbolic function (Monod equation), relate the specific growth rate, μ, to substrate concentration. The investigative purpose herein was to determine the effect of substrate concentration on exponential growth rate with special attention to bacterial cells growing at substrate concentrations below μ_{max}. These metabolic growth "constants," sludge yield (Y), maximum exponential growth rate (μ_{max}),

and "saturation constant" (K_s), are applicable as descriptors for the growth of microbial populations in discontinuous as well as continuous systems in the laboratory industrial fermentor or the natural environment. They are especially important in considering models for activated sludge systems which are designed to be operated as steady-state, completely mixed, continuous flow systems. The theoretical developments assume steady-state conditions in the reactor (i.e., the aeration unit) and for this to be so, the microbial population should always be in exponential growth and the specific growth rate is related to the dilution rate, a hydraulic factor in continuous flow wastewater treatment systems that is subject to engineering control.

The "endogenous" or autodigestive phase, was examined in these studies because the operational biochemical stability and efficiency of the activated sludge-total oxidation process which employs 100 percent sludge return and no sludge wastage relies on a metabolic balancing of autodigestion and new sludge synthesis, thus negating biological solids accumulation to an extent which would require sludge wastage.

Thus, in this investigation, the overall aim was to study and gain insight into quantitative description of the entire cycle of growth and decay of a heterogeneous (i.e., natural) bio-mass.

CHAPTER II

LITERATURE REVIEW

The relationship between the yield coefficient, Y, and the chemical constituents of the organic carbon source metabolized, the nature and characteristics of the waste, and the type of organism, is a topic of which there has been considerable speculation and discussion, both in the basic science and engineering literature.

The concept that the amount of sludge produced during biological respiration and synthesis varies with the chemical nature of the waste was reported by Placak and Ruchhoft (1), Sawyer (2), and McCabe and Eckenfelder (3). Placak, et al., investigating the utilization of pure organic substrates, reported sludge yields on carbohydrate wastes in the range of 65 to 85 percent. Sawyer's studies of various carbon sources showed conclusively that yield values of 50 to 60 percent can be expected. McCabe and Eckenfelder related cell yield to the "fraction of BOD removed which is synthesized to new sludge," and taking into account the amount of "self-metabolism," reported a yield value of 0.7 for glucose. Similar results are reported by Wuhrmann (4) for the compounds glucose, peptone, and lactate.

The theory that the synthesis of a compound is fixed, i.e., is independent of the nature of the organic matter assimilated, appears to have been proposed by Helmers, et al. (5), Heukelekian, et al. (6),

Hoover, et al. (7), and McKinney (8). Helmers, et al. reported that the rate of activated sludge growth is proportional to the BOD reduction of the waste and independent of the nature of the waste load. Heukelekian, et al. showed that sludge production can best be expressed by relating the volatile suspended solids in the mixed liquor to the BOD of the waste feed. Hoover, et al. researched the assimilation of a dairy waste and, using COD as a parameter of waste purification, found the microorganisms in an activated sludge to oxidize 32 to 43 percent of the carbon, with the remainder incorporated into the cells. McKinney concluded that two-thirds of the ultimate oxygen demand of the organic matter is employed for cellular synthesis. However, in a later publication, Burkehead and McKinney (9) found that the energy synthesis reactions are substrate-dependent.

That portion of the substrate channelled into synthesis has also been described using thermodynamic concepts and parameters. Bauchop and Elsdon (10) proposed a different approach to sludge synthesis by relating cell yield to the calculated theoretical production of ATP from an energy souce during metabolism by several anaerobic cultures. Servizi and Bogan (11), employing the theory of Bauchop and Elsdon, further postulated a concept of constant proportionality of ATP production to the free energy released through substrate oxidation, and stated that for carbohydrates, the cell yield was 0.38 g of cells/gm COD removed in aerobically growing systems.

McCarty (12) recognized that relation between free energy, ATP, and cell yield must take into cognizance two operations: the generation of the ATP and its coupling to useful synthesis. He developed a formula which relates cell yield to free energy and contains transfer

efficiency proportionality constants. For acetic acid, a yield of 0.41 g of dry sludge solids/gm COD removed, was reported.

Operating continuous flow reactors with pure and mixed cultures at various dilution rates, Hetling, et al. (13) found that effective yield coefficients are not constant with substrate and organism. For sodium acetate, he showed a true yield coefficient (effective yield coefficient corrected for endogenous "heterogeneous metabolism") for Pseudomonas fluorescens of 0.19, and for E. coli, 0.27 mg cells/mg substrate COD. These values illustrate the variability of cell yield.

A review of the energy concepts of oxidative assimilation by Servizi and Bogan, McCarty, and McKinney, is presented by Burkehead and McKinney (14). In addition, the relationship between oxygen and heat of reaction is shown to support the concept of energy and synthesis on an oxygen equivalence basis.

Payne (15) has made a review of yield factors involved in 1) yield and ATP generation, 2) yield and electron availability in substrate, and 3) yields per kilocalorie of total energy taken by both assimilation and dissimilation from the medium during growth.

In reviewing the energetic behavior of the growing microbial cell, Forest (16) points out that during oxidative metabolism, the knowledge of the efficiency of oxidative phosphorylation is incomplete.

It should be noted that speculation on the number of moles of ATP that can be generated from aerobic catabolism represents the greatest impediment to experimental determinations of Y_{ATP} for aerobes. It also seems that a prediction of yield for microorganisms in activated sludge and quantitation of the transformation of the energy of substrate molecules into cells and end products by bacterial metabolism based solely

on thermodynamic considerations of the waste is a rather fruitless exercise in attempts to estimate or predict cell yield.

Operating batch activated sludge systems for 90, 90, 65 days at several different initial solids concentrations and feeding glucose as sole carbon source, Rao and Gaudy (17) observed an average cell yield of 65 percent with a statistical range of yield from 48 to 82 percent, and stated that variance in cell yield was due to changes in predominance of species in the population.

Gaudy and Ramanathan (18) have compiled cell yield data over a period of eight years for acclimated natural microbial populations of sewage origin grown on glucose as sole growth-limiting factor in batch and continuous flow systems. A statistical analysis shows for batch studies a mean yield of 61.9 percent for 118 experiments with a range of 36 to 88 percent, and for continuous flow experiments, a mean yield of 49.9 percent for 81 experiments with a range of 32 to 69 percent. From this study they concluded that cell yield is subject to ecological variance in mixed microbial populations.

Under defined and comparable conditions with methodology and parameters the same for all determinations of sludge yield, Ramanathan and Gaudy (19) presented a statistical summary of sludge yield values for heterogeneous populations of sewage origin grown on various carbon sources. From six batch experiments with acetate, a mean yield of 41.2 percent was obtained with a range of 26 to 53 percent.

During substrate removal and biological growth, the physiological growth parameters for cells, maximum specific growth rate (μ_{max}), saturation constant (K_s), and cell yield (Y), are very important kinetic "constants" in the development of kinetic models for pure and heterogeneous microbial cultures.

The concept of a single continuous function of decreasing slope (hyperbolic function) proposed by Monod (20), which is discussed extenisvely later, is often used when describing the relationship between exponential growth rate (μ) and substrate concentration. However, Garret and Sawyer (21) proposed using two different functions to describe the relationship between specific growth rate and substrate concentration; one function for the lower range of substrate concentration where growth rate is assumed to be linear, and one for the higher range of substrate concentration where growth rate is considered to be constant and independent of substrate concentration.

McCabe and Eckenfelder (3) have concluded that the critical substrate concentration at which the growth rate approaches a maximum varies with cell concentration. It is known here that the two should be independent.

Schulze has employed a modification of a hyperbolic equation proposed by Teissier (22). Schulze employed data from a pure culture study using Escherichia coli (23) and data obtained from wastewater treatment plants (24). He concluded that Teissier's modification should yield satisfactory results.

Gaudy, Ramanathan, and Rao (25) compared the Monod, Teissier, and Moser (26) models for growth kinetics of natural populations in continuous flow completely mixed reactors, and concluded that the Monod model best described the relationship between μ and S. They also found the yield coefficient (Y) to be lower in batch operated systems than in continuous flow systems. They also found that K_s values of 75 to 125 mg/l and μ_{max} values of 0.5 to 0.6 hr^{-1} with a yield coefficient of about 0.6 was usually observed. In a continuation of their research,

Ramanathan and Gaudy (27) have presented equations and computational analyses for a steady-state model that describes the kinetic behavior of an activated sludge system. In their model, recycle cell concentration, X_R, is employed as a controllable operational constant. Their model differs rather drastically from that recommended by Herbert, et al. (28)(also see Herbert [29]), in which the recycle sludge concentration factor, C, is the operation constant. This constant in Herbert's model is the ratio of biological solids in the recycle flow to biological solids, X, in the reactor (i.e., $C = X_R/X$).

Peil and Gaudy (30), using mixed microbial cultures of sewage origin growing on various substrates including municipal sewage, found the rectangular hyperbola to provide satisfactory description of the relationship between specific growth rate and substrate concentration.

More recently, Gaudy, Obayashi, and Gaudy (31) have presented data which is in aposition to the Monod theory. This article is discussed later in greater length.

After accumulation of biological solids during the initial substrate removal and growth phases resulting from microbial metabolism of the waste to cellular compounds and end products, it becomes necessary to dispose of this synthesized bio-mass. The activated sludge-extended aeration process is a system for treatment of the waste and ultimate disposal of such excess organic matter produced during treatment. The operation of a "total oxidation" system implements the aerobic dissipation of sludge by "endogenous" or autodigestive metabolism, and a phase of this investigation herein is directly concerned with prolonged endogenous aeration of an accumulated bio-mass.

Hoover, et al. (32) researched the aerobic autodigestion of

bacterial cells fed skim milk solids and derived an average endogenous respiration rate of 3 to 4 ppm O_2/hr for a sludge produced by oxidation of 1000 ppm skim milk solids. In comparison, the O_2 consumption rate of the same organisms during the initial period of assimilation was 40 to 45 ppm O_2 hr, indicating considerably less energy utilization during endogenous metabolism. In a later publication, Porges, et al. (33) noted that for the total auto-oxidative digestion of 500 ppm cell substance produced from 1000 ppm skim milk solids, a time of approximately 160 hours was necessary.

Forney and Kountz (34), using a continuous flow system treating a skim milk waste, noted that "total bio-oxidation" is possible and that a solids equilibrium was attainable.

Operating daily batch-fed activated sludge systems on sodium acetate as substrate with 100 percent sludge recycle over a period of 35 days, Symons and McKinney (35) refuted the concept of total oxidation. They observed an accumulation of volatile biological solids during the entire time period of each experiment. They concluded that the accumulated material which was observed to consist in part of extracellular polysaccharide, was non-oxidizable material, i.e., biologically inert material. They concluded that sludge wastage is necessary for successful biochemical performance of activated sludge systems.

Kountz and Forney (36) later rejected the concept of total oxidation on the principle that it is "not possible within reasonable times" and will require excess size of aeration tanks in waste treatment systems. After operating a continuous flow activated sludge system for six months, they concluded that a residual material remained comprising 20 to 25 percent of the sludge synthesized. They further concluded an

endogenous loss of two percent/day and a buildup of an inert fraction at the rate of 0.6 percent/day with respect to the total weight of the organisms, occurred in the system.

In later research, Jasewicz and Porges (37)(38) studied the oxidation and assimilation of whey wastes by aerobic organisms, and reported that sludge accumulation occurred. They attested positively to the theoretical possibility of a total oxidation plant, but stated that actual operational conditions will not facilitate total sludge oxidation and occasional sludge wastage is necessary.

In a continuing effort to define the theoretical basis of total oxidation and endogenous respiration, Washington and Symons (39) researched the accumulation of volatile biological solids grown on various organic compounds under batch-fed and continuous flow completely mixed systems. They concluded that there would be an organic sludge buildup of 10 to 15 percent of the ultimate BOD of the substrate removed for carbohydrate or fatty acid feeds. Investigating the extent of degradation of cellular organic polymers during endogenous aeration, they concluded that the biologically inert organic solids that accumulate and are resistant to degradation during "endogenous" metabolism, either by the organism which produced the substance or by a predator, are mainly polysaccharide in content (47 to 56 percent); protein (39 to 47 percent), and fats (3 to 8 percent), comprise the remainder.

McWhorter and Heukelekian (40) studied once-fed (glucose) batch activated sludge systems over an extended aeration period of 25 days. After approximately ten days, the oxidation rate of the sludge increased very slowly. The cell mass remaining represented 40 percent of the maximum accumulation of sludge produced, or 12 percent of the

theoretical oxygen demand of substrate glucose. The chemical composition of the remaining cell mass was not determined directly, but was theorized to be carbonaceous in content.

In a recent research investigation, Thabaraj and Gaudy (41) operated once-fed long-term batch activated sludge systems growing on either glycerol or sorbitol as sole carbon source and growth-limiting nutrient for extended durations of endogenous metabolic activity. They presented evidence that total aerobic autodigestion of an accumulated cell mass is definitely possible.

The theoretical soundness of the extended aeration process is usually questioned by researchers who contend that there has to be an inactive fraction which cannot be metabolized or cannot metabolize the influent waste and therefore build up in the system and necessitate sludge wastage and a possible breakdown of the biochemical mechanisms in the system. Recently, extensive research concerning the availability of certain cellular organic molecules and biopolymers which can exist as this "inert" fraction as a possible food or nutrient source, has been completed by Obayashi (42). Specifically, investigative experiments pertained to the biodegradative nature of extracellular polysaccharide and sonicate (the soluble portion of the cells released after breakage of the cell walls and capsular layer). Short-term batch experiments were conducted using polysaccharide harvested from various pure culture species and cell sonicate was also supplied as sole carbon source. In a recent article, Obayashi and Gaudy (43) have shown bacterial extracellular heteropolysaccharide does not constitute biologically inert fraction. Also, in the Oklahoma State University bioenvironmental research and engineering laboratories, Yang (44) has

completed a four-year study in which a continuous flow pilot plant of the extended aeration process was operated with and without an assist by chemical hydrolysis of portions of the recycled sludge. In recent articles relating to these studies, Gaudy, et al. presented data showing cyclic periods of biological solids accumulation and de-accumulation in the aeration unit, and pointed out that during times of de-accumulation, the biochemical efficiency remained high. They also found that there was not a continual buildup of an inactive fraction of biological solids, and at no time was there any failure of biochemical mechanisms of purification. However, it was observed that at times, cell concentration became so great that it caused settling problems in the clarification chamber. A solution to this problem was to chemically hydrolyze a portion of the return sludge, and cycle it to the aeration chamber with the regular influent substrate. They named this modification of the process, the "hydrolytic assist." The pilot plant was operated for a one-year period in which portions of the biological solids were periodically withdrawn, hydrolyzed, and recycled, and it was found that this process modification is operationally feasible and provides engineering control over the concentration of organisms in the total oxidation or extended aeration process (45).

CHAPTER III

MATERIALS AND METHODS

A. Experimental Protocol

Mixed microbial cell populations were developed on acetic acid as the sole carbon source from an initial sewage seed obtained from the primary clarifier effluent of the municipal wastewater treatment plant in Stillwater, Oklahoma. The composition of the synthetic waste (growth medium) is given in Table I.

TABLE I

CONCENTRATIONS OF INORGANIC COMPONENTS OF SYNTHETIC WASTE PER 1000 mg/l COD OF ACETIC ACID

Compound	Concentration
$(NH_4)_2SO_4$	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
1.0 M potassium phosphate buffer, pH 7.0	55 ml
Tap water (for trace elements)	100 ml
Distilled water	to volume

The cells were cultivated in batch reactors (500-ml Erlenmeyer flasks). The reaction liquor consisted of growth medium and an inoculum of sewage seed. Aeration was provided by a shaker apparatus operating at 100 oscillations/min. Daily, a portion of the mixed liquor was transferred to a flask containing fresh growth medium. Acclimation of the cells to the substrate for four days was precedent to experimental use. All experimental procedures were conducted at room tempterature (21 $\pm$ 2^{o}C), and for each batch aeration experiment performed, a different initial sewage seed was employed.

1. Long-term Batch Experiments

After the acclimation period, the organisms were harvested and a portion of the acclimated cell suspension was utilized to inoculate the synthetic waste. The remaining volume of seed culture was used for growth rate experiments (described later). The long-term batch experiments were accomplished initially in two- and four-liter Erlenmeyer flasks with a substrate concentration of 1000 mg/l acetic acid. As the volume decreased, the mixed liquor was transferred to one- and two-liter Erlenmeyer flasks. Compressed air saturated by passage through a water trap was supplied through sintered glass diffusers. At the time of inoculation, a portion of the suspension in the activated sludge system was obtained for initial measurement of chemical oxygen demand, biological solids concentration, and in later experiments, cell protein and cell carbohydrate. Throughout the duration of the experiment, suspensions of the mixed liquor were obtained for measurement of the physical, chemical, and biochemical parameters described above. Also the initial optical density was recorded. During each experiment, frequent measurement of optical density permitted construction of a growth curve

which was used as a guide in selecting sample times. Starting with the next day, the volume of the mixed liquor was measured prior to sampling and the loss of water due to evaporation was made up to the correct volume with distilled water. The pH was 7.0 ($\pm$ 0.15) in all of the systems.

2. Growth Rate Studies

In the growth rate experiments, cells from the batch reactors were inoculated into flasks (250-ml Erlenmeyer) containing fresh growth medium, essential salts (as described earlier), and various concentrations of acetic acid as the sole carbon source and growth-limiting nutrient.

Seven concentrations of carbon source within a range of 60 to 1000 mg/l acetic acid were aerated on a shaker (Eberbach, 110 oscillations/min), and the experiments were performed concurrently with experiments described above. Each flask was inoculated with 2.5 ml of cell suspension from the batch reactor and minimal growth medium to a volume of 50 ml. The pH of all systems was seven. For all experiments, growth of biological solids was measured by optical density.

B. Analytical Methods

1. Biological Solids

The weight of biological solids was determined gravimetrically by filtration of the mixed liquor samples through membrane filters (0.45 μ pore size, Millipore Filter Corp., Bedford, Mass.). The following procedure was employed for the measurement of suspended biological solids: Filters were placed in aluminum pans weighing approximately 1.3 grams. The pans were placed in a drying oven for one hour at a temperature setting of 103^{o}C, and then placed in a desiccator for cooling. After

cooling, the pans were weighed. Known volumes of mixed liquor were then filtered with the aid of a vacuum pump. For samples which were difficult to filter, a centrifuge (Sorvall Superspeed Centrifuge, type SS-1A, Ivan Sorvall, Inc.) was used to reduce the time of filtration. The samples were centrifuged (10,000 rpm) for several minutes prior to filtration. The supernatant was carefully filtered first, and then the pellet of solids which was formed was removed with the aid of a metal spatula and placed on the filter. After complete filtration, the filters were returned to the pans, placed in a drying oven at a temperature of 103^{o}C for one hour, cooled in a desiccator, then weighed to determine the biological solids concentration.

2. Chemical Oxygen Demand

The COD of the membrane filtrate was determined in accordance with Standard Methods (46). Mercuric sulfate and silver sulfate were used for all COD determinations.

3. Protein Content of Biological Solids

Protein was measured using the Folin-Ciocalteu reagent as described by Ramanathan, Gaudy, and Cook (47). The analyses were performed on cell suspensions which were homogenized by sonic oscillation. The standard used was crystalline bovine plasma albumin, Grade A (Calbiochem, Los Angeles, California).

4. Total Carbohydrate Content of Biological Solids

Carbohydrate was measured, using the anthrone method as outlined by Ramanathan, Gaudy and Cook (47). Analyses were performed on aliquots of homogenized cell suspensions and the standard used was reagent grade glucose.

CHAPTER IV

DATA ANALYSIS

A. Cell Yield

All sludge yield values were determined by calculating the slope of the straight-line plot of biological solids versus substrate (COD) removed for each long-term batch study. A statistical analysis was performed on the values for cell yield (Y), maximum growth rate (μ_{max}), and saturation constant (K_s). The statistical parameters, sample mean ($\bar{x}$), standard deviation (s), coefficient of variance (CV), and 95 percent confidence limit (CL) were calculated from the following mathematical expressions (48):

1. Sample mean ($\bar{x}$)

$$\bar{x} = \frac{\Sigma_i X_i}{n} \tag{1}$$

where $\bar{x}$ = sample mean

$\Sigma_i X_i = X_1 + X_2 + \ldots + X_i + \ldots X_n$

n = total number of observations

2. Standard deviation (s)

$$s = \sqrt{\frac{\Sigma(X - \bar{x})^2}{(n - 1)}} \tag{2}$$

where

s = standard deviation

$X = X_1, X_2, X_n$

$\bar{x}$ = sample mean

n = number of samples

3. Coefficient of variance (CV)

$$CV = \frac{100\ s}{\bar{x}} \qquad (3)$$

where

CV = coefficient of variance

s = standard deviation

$\bar{x}$ = sample mean

4. 95 percent confidence limit (CL)

$$0.95 = P\left[\bar{x} - \left(t_{0.05} \cdot \frac{s}{\sqrt{n}}\right) \leq \mu \leq \bar{x} + \left(t_{0.05} \cdot \frac{s}{\sqrt{n}}\right)\right] \qquad (4)$$

where

$\bar{x}$ = sample mean

s = standard deviation

n = total number of observations

t = "Student's t"

μ = population mean

B. Growth Kinetics

For each growth experiment, optical density vs. time for each initial COD concentration was plotted on semi-logarithmic graph paper. When the population is in exponential growth, this plot will yield a straight line portion, the slope of which is the exponential growth rate constant (μ) in agreement with the following equation which describes the increase in cell mass during logarithmic growth of a bacterial culture:

$$\frac{dx}{dt} = \mu \cdot X \tag{5}$$

where X is the dry weight of cells per unit volume at time, t, and μ is the specific growth rate. Integration of this expression yields the growth equation

$$\mu(hr^{-1}) = \frac{\ln \Delta X}{\Delta t} \tag{6}$$

The specific growth rate values with the corresponding initial substrate concentrations for each growth system were then plotted according to a Lineweaver, Burk representation (this is a graph of $1/\mu$, ordinate vs. 1/S abscissa). A curve of best fit for this plot is a straight line, the slope equaling K_s/μ_{max}, with the Y intercept being $1/\mu_{max}$.

A plot of μ versus S (Monod plot) was also constructed for each growth rate experiment. It has been concluded by Monod (20) that best representation of the relationship between specific growth rate, μ, and substrate concentration, S, is a rectangular hyperbola in accordance with the equation

$$\mu = \mu_{max} \frac{S}{K_s + S} \tag{7}$$

In this equation, the maximum growth rate constant (μ_{max}) is defined as that growth rate at which a further increase in the growth-limiting nutrient has no effect on the specific growth rate, μ, and K_s, the saturation constant, is the substrate concentration at which $\mu = 0.5\ \mu_{max}$. Examination of equation (7) indicates that exponential growth does not occur unless the growth-limiting nutrient is in excess and this substrate concentration corresponds to μ_{max}. This means that in a microbial growth system where substrate molecules are not in excess, the

specific growth rate constant (μ) is so sensitive to substrate concentration that a change in substrate concentration, due to bacterial metabolism, will cause a change in the specific growth rate (μ). The Monod equation is widely used by microbiologists for studies on the kinetics of bacterial growth, and has also been applied by bioenvironmental engineers to the study of "steady-state" growth of mixed microbial populations for possible use in biological wastewater treatment plant design.

In contrast to Monod's theory, Gaudy, Obayashi, and Gaudy (31) have shown exponential growth (as determined by slope of the linear portion of a plot of optical density [logarithmic] vs. time on semi-logarithmic paper) can exist at substrate levels below those needed to attain μ_{max}. They have hypothesized that a biological cell can control internal concentrations of substrate, within certain limits set by the initial substrate concentration, S_o, so that while the external substrate concentration may change, the internal substrate concentration remains relatively constant and sets the exponential growth rate.

The Monod graph was constructed to see how well the data reported herein fit the hyperbolic relationship of specific growth rate vs. substrate concentration. All numerical values of maximum growth rate (μ_{max}) and the saturation constant (K_s) were calculated from each Lineweaver, Burk plot.

CHAPTER V

RESULTS

The research investigation is concerned with a kinetic and metabolic study of acetic acid metabolism and the effect of substrate concentration on exponential growth. In section I, the results during substrate removal and endogenous phases for twenty long-term once-fed batch activated sludge experiments using acclimated natural microbial populations of sewage origin are presented. In section II, the results of cell yield analysis and kinetic treatment of data from growth rate experiments are shown. In section III, results for endogenous metabolic activity and total oxidation of activated sludge mixed microbial populations are presented.

A. Primary Data

Figures 1-20 show the parameters measured during the metabolism of acetic acid.

1. Metabolic Response of Acclimated Natural Microbial Cultures Growing on Acetic Acid (Figures 1-7)

The substrate utilization patterns for experiments 1-7 (Figures 1-7) are similar. COD removal and biological solids accumulation appeared to follow the S-shaped patterns typical of most growth experiments. COD removal and solids growth were plotted on semi-logarithmic

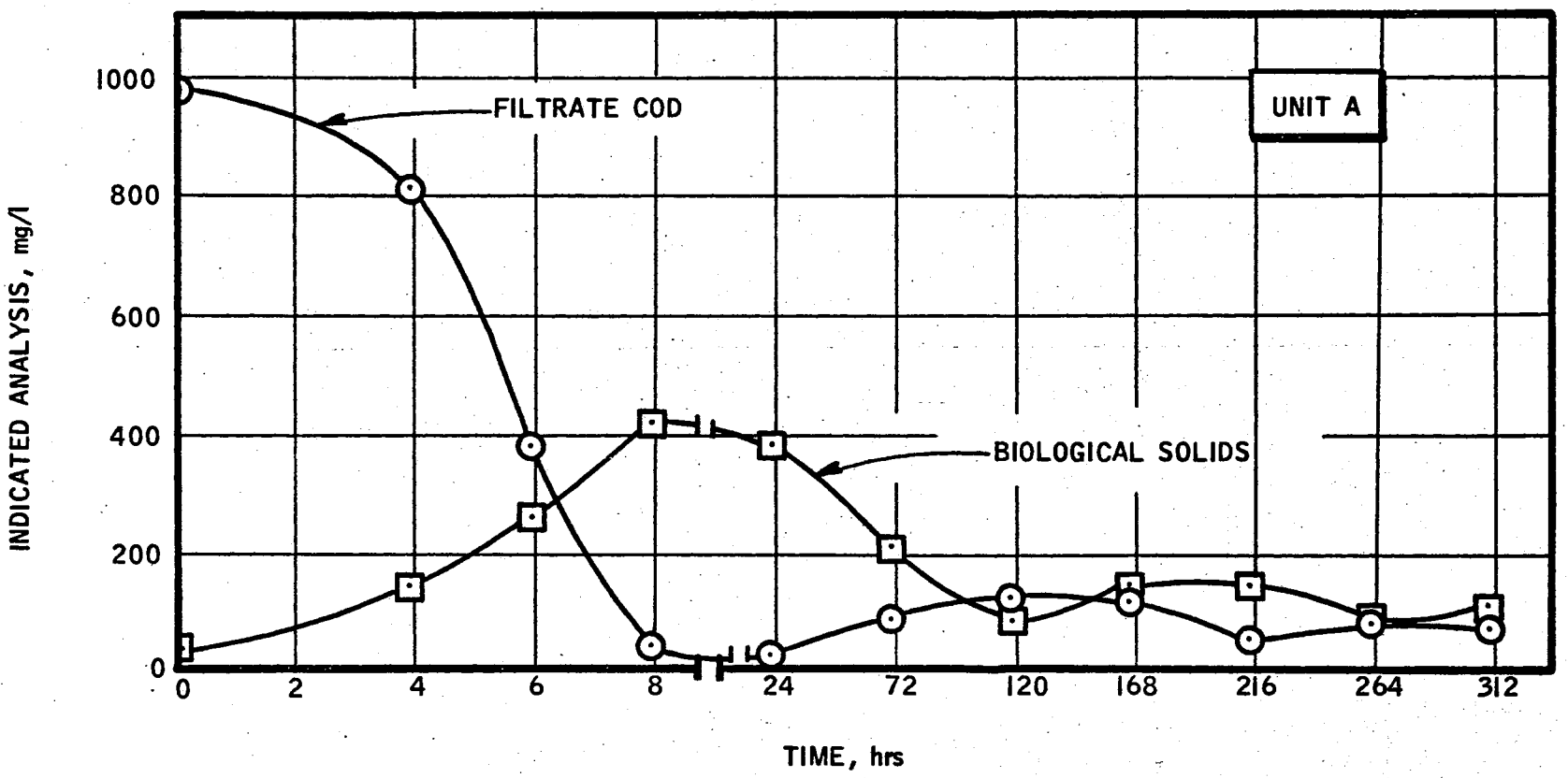

Figure 1. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 1.

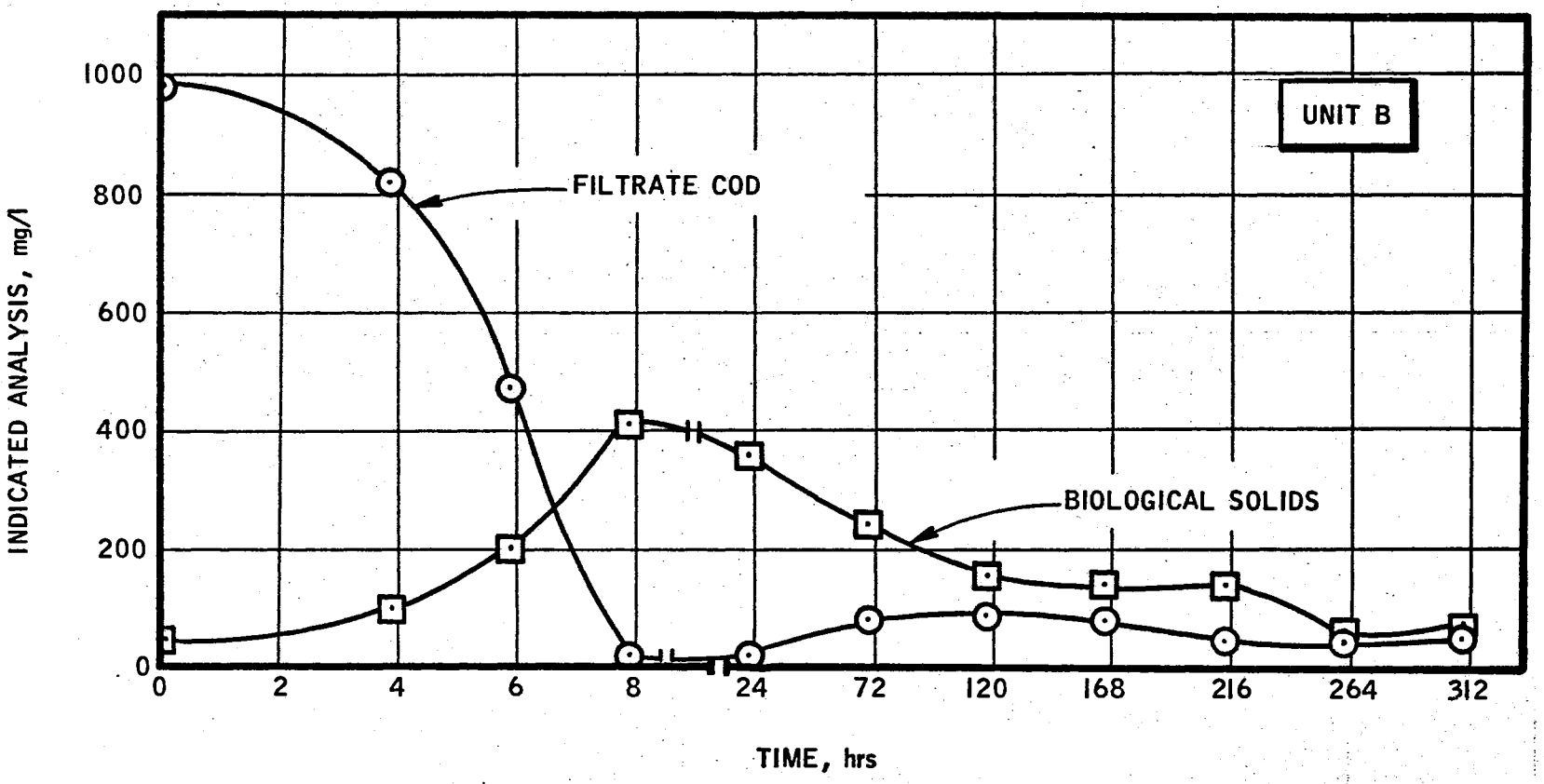

Figure 2. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 2.

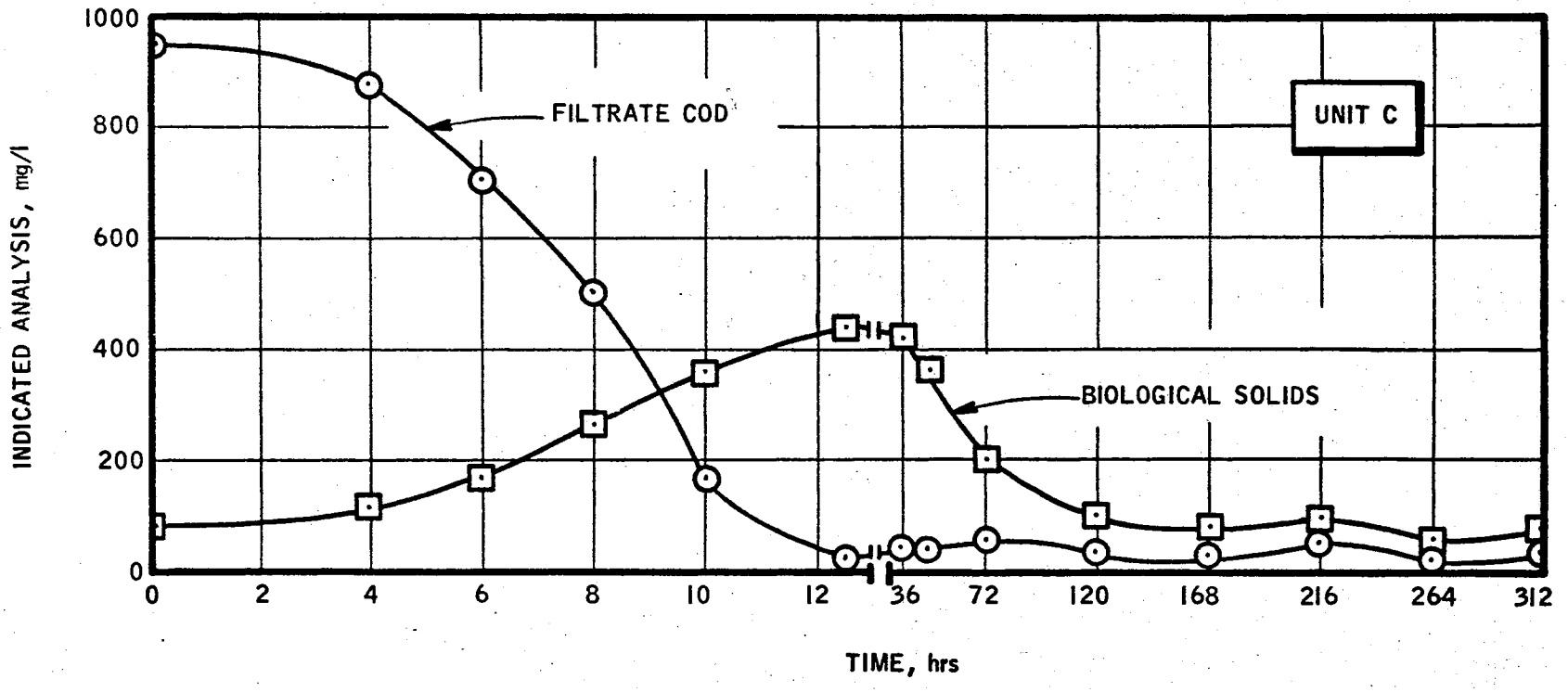

Figure 3. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 3.

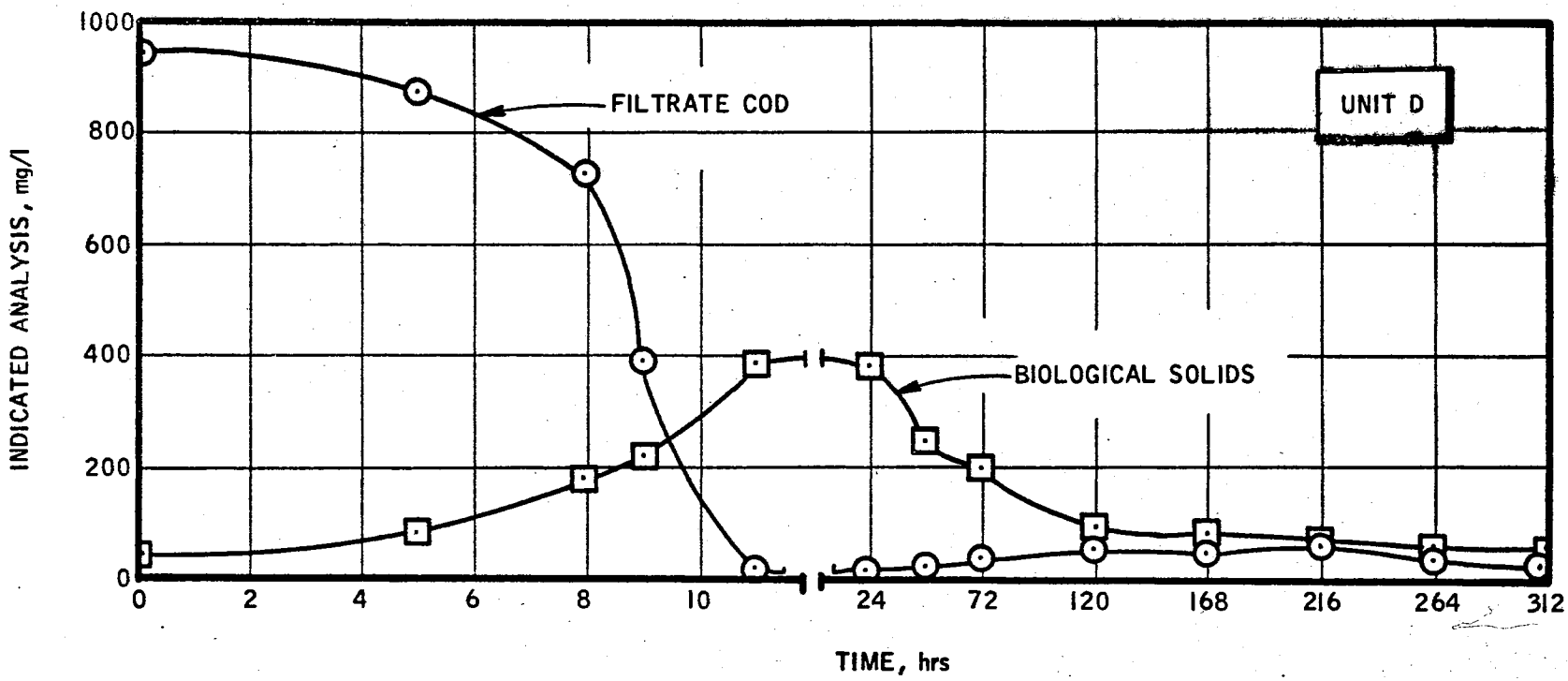

Figure 4. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 4.

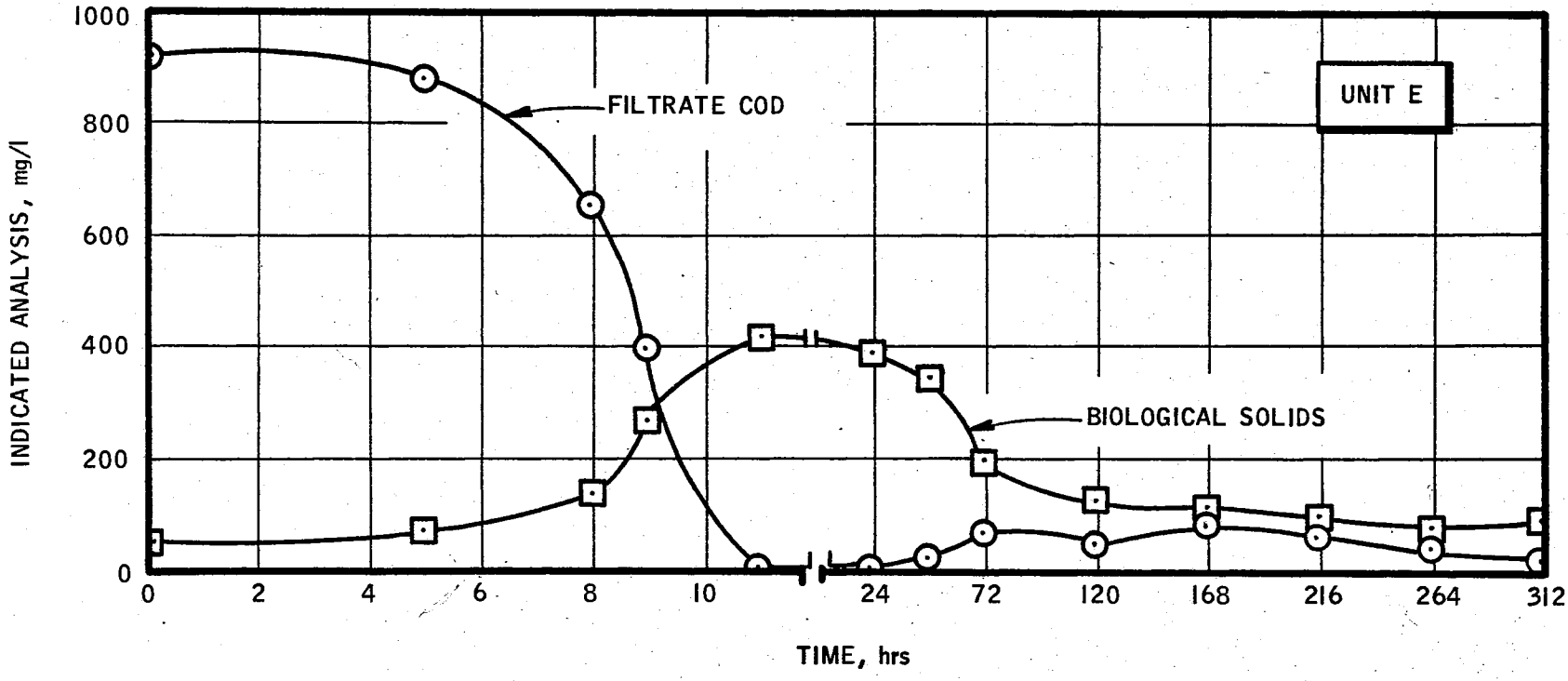

Figure 5. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 5.

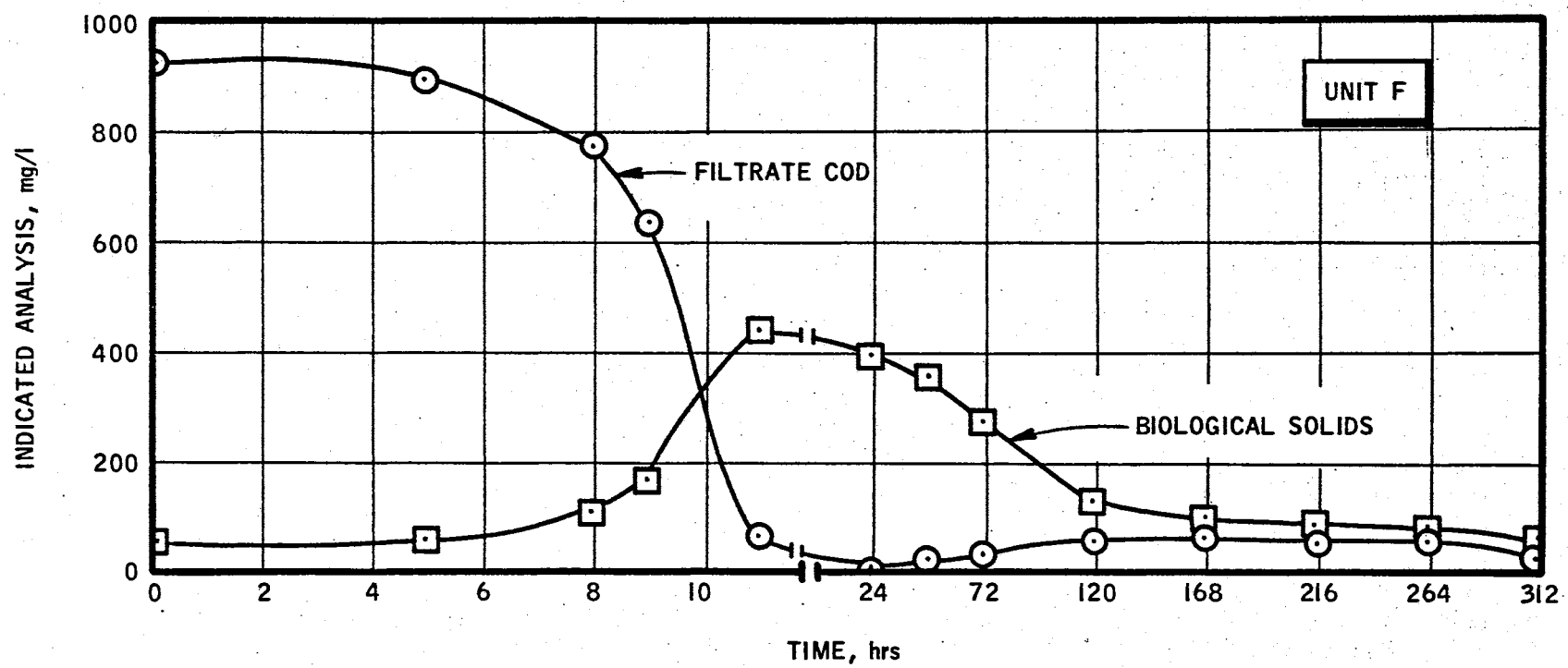

Figure 6. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 6.

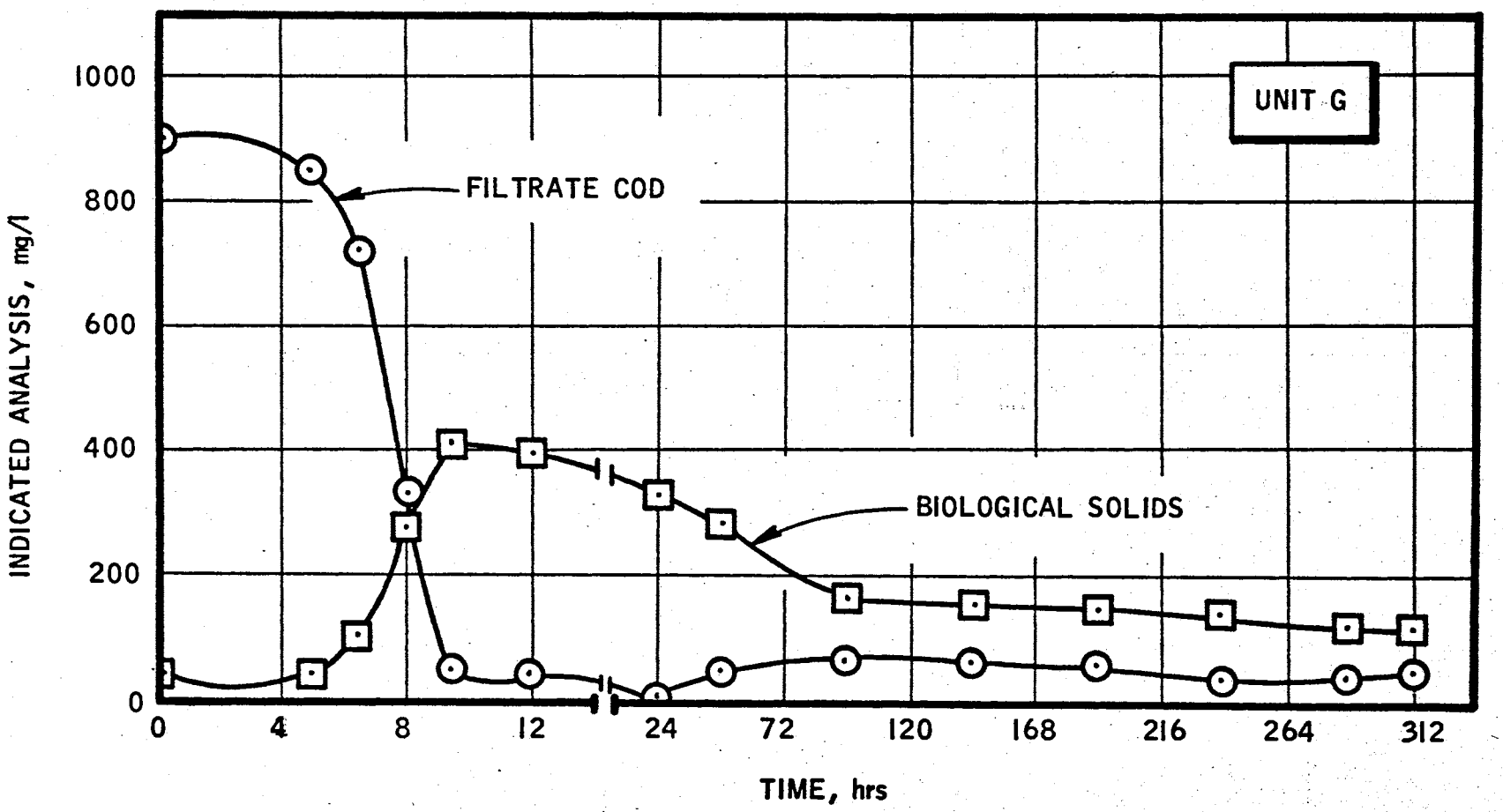

Figure 7. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 7.

coordinates, and distinct exponential phases were observed in all cases. The biochemical efficiency, i.e., the percent substrate removal during the waste purification phase for the systems, ranged from 93 to 99 percent. The duration of the purification phases varied from 8 to 12.5 hours. It can be seen in Figures 1-5 that maximum solids accumulation occurred at time of maximum removal of the exogenous substrate. However, in Figures 6 and 7, the lowest amount of soluble organic matter was observed at 13 and 14.5 hours, respectively, i.e., somewhat after the time of maximum sludge synthesis. The percentage of carbon source (as COD) that was channelled into cellular synthesis, or cell yield, for experiments 1-7 (Figures 1-7) varied from 37.5 to 50 percent.

Endogenous oxidation patterns of the biological solids in the activated sludge systems (Figures 1-7) were similar. At first, the rate of endogenous oxidation proceeded reasonably fast but later, after approximately 120 hours, approached zero. The solids synthesized earlier during growth in experiments 1 and 7, did not appear to be as readily (totally) utilized during the endogenous phase as in the other experiments. It can be seen from Figures 2 and 5 that almost "total oxidation" of the accumulated solids occurred during the endogenous (autodigestion) phase. The results shown in Figures 3, 4, and 6 show total aerobic autodigestion of the synthesized sludge mass. The total filtrate COD remaining in experiments 1-7 (Figures 1-7) was relatively constant and of low concentration throughout the entire endogenous phase. Table II is a summary of characteristics of acetic acid systems 1-7 at the time of removal of exogenous substrate.

TABLE II

SUMMARY OF CHARACTERISTICS OF ACETIC ACID SYSTEMS 1-7 AT THE TIME OF REMOVAL OF EXOGENOUS SUBSTRATE

					Biochemical Efficiency	Sludge Synthesis			Physiological Growth Constants	
Exper. No.	Fig. No.	Time (hrs.)	Residual mg/l	Removed mg/l	%	Total mg/l	mg/l	Yield Percent	μ_{max} hr^{-1}	K_s mg/l
1	1	8	43	930	96	420	390	43		
2	2	8	27	959	97	415	367	40		
3	3	12.5	24	926	97	443	360	38		
4	4	11	11	932	99	395	335	37.5	*0.413	99
5	5	11	5	923	99	425	370	40	*0.477	99
6	6	11	69	858	93	440	385	50	*0.366	61
7	7	9.5	59	846	94	420	380	46	0.980	81.5

*Not included in statistical analysis.

2. Metabolic Response and Biochemical Composition of the Bio-mass Growing on Acetic Acid (Figures 8-20)

The substrate removal and sludge synthesis patterns for experiments 8-20 (Figures 8-20) were, in general, similar to those observed for the previous experiments. The activated sludge systems exhibited biochemical efficiencies in the range of 80 to 99 percent, and the lengths of substrate removal phases varied from 7 to 10.5 hours with the exception of experiment 20 (Figure 20), which lasted 27 hours. Cell yield values of 35 to 45.7 percent were obtained in experiments 8-20 (Figures 8-20).

From the data of Figures 8-20 and Table II, the latter presenting a summary of characteristics of acetic acid systems 8-20 at the time of removal of exogenous substrate, synthesis of cellular carbohydrate at the time of maximum removal of exogenous substrate ranged from 3.0 to 9.7 percent of the accumulated biological solids. Protein synthesis paralleled the acclimated biological solids curves and amounted to 41.1 to 63.5 percent of the accumulated cell mass.

The aerobic auto-oxidation of the accumulated biological solids followed patterns similar to earlier experiments (see Figures 1-7). The rate of autodigestion was fairly rapid at first, then gradually approached zero during prolonged endogenous aeration conditions. The carbohydrate content of the cells remained relatively low during the entire period of endogenous metabolism. Oxidation of proteinaceous molecules seemed to be the main source of endogenous substrate. Experiments 8-10 (Figures 8-10) show "total oxidation" of the sludge mass accumulated during growth. Various degrees of aerobic autodigestion of solids accumulated during the substrate removal phase were observed in

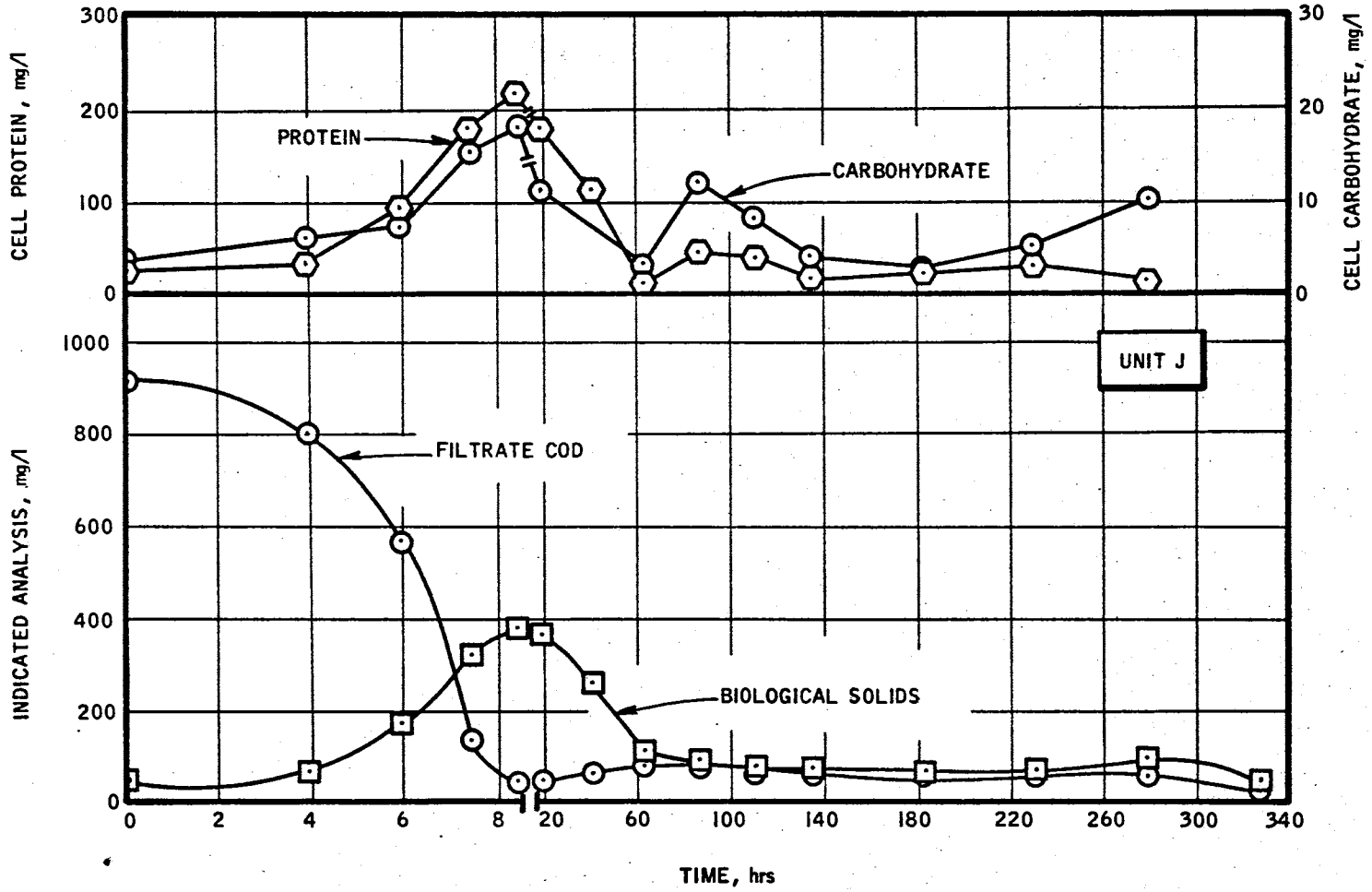

Figure 8. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 8.

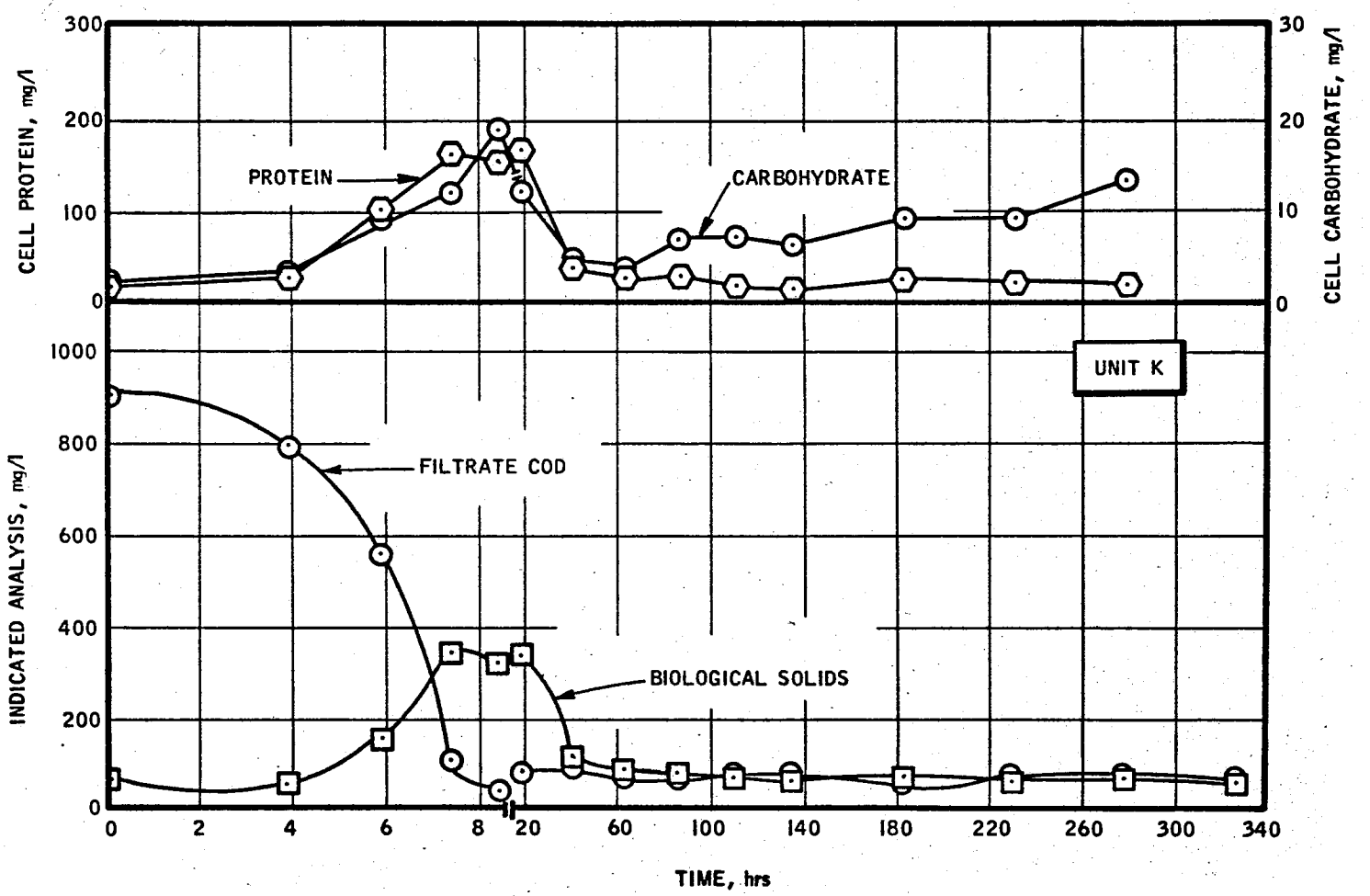

Figure 9. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 9.

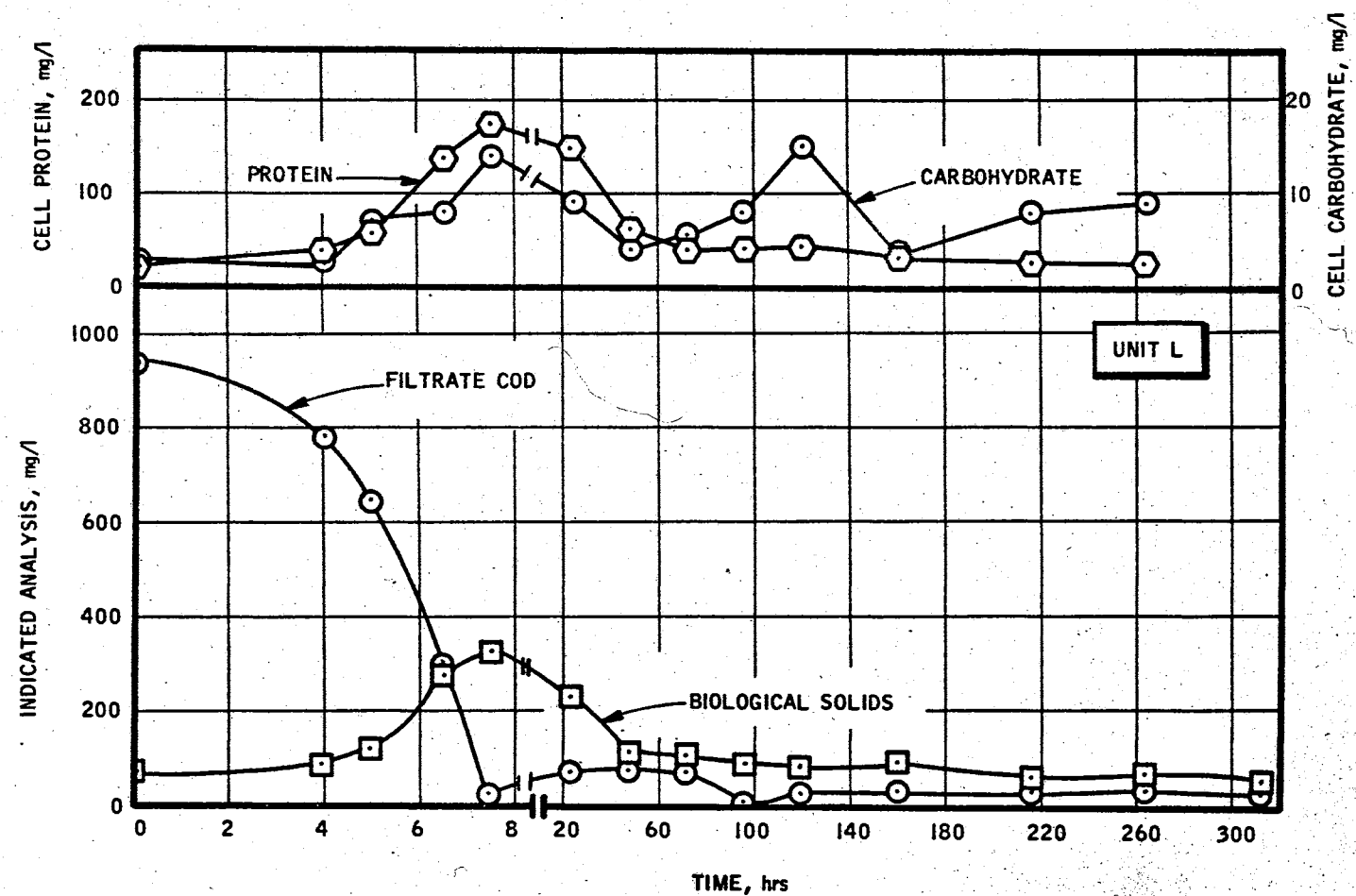

Figure 10. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 10.

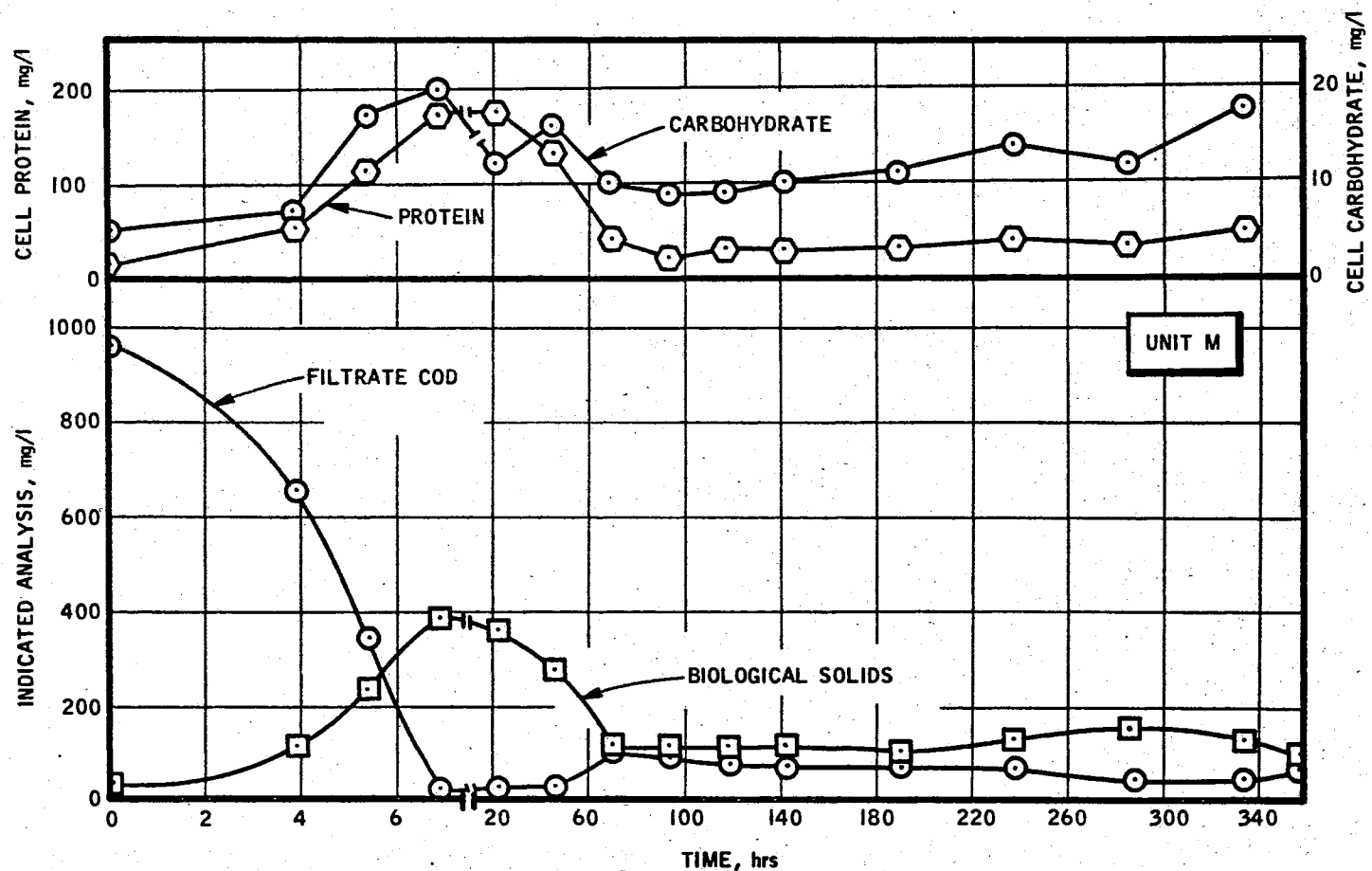

Figure 11. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 11.

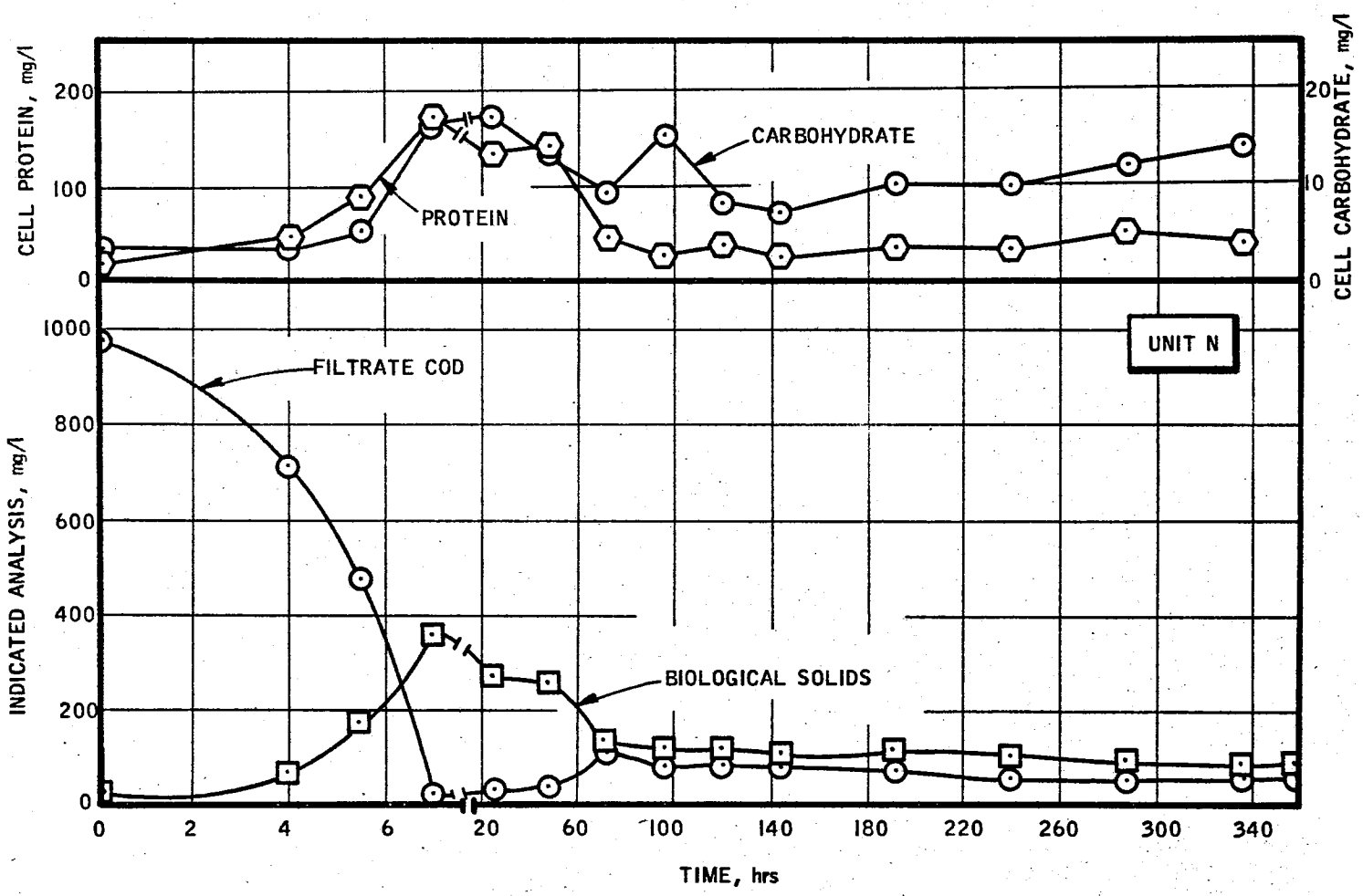

Figure 12. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 12.

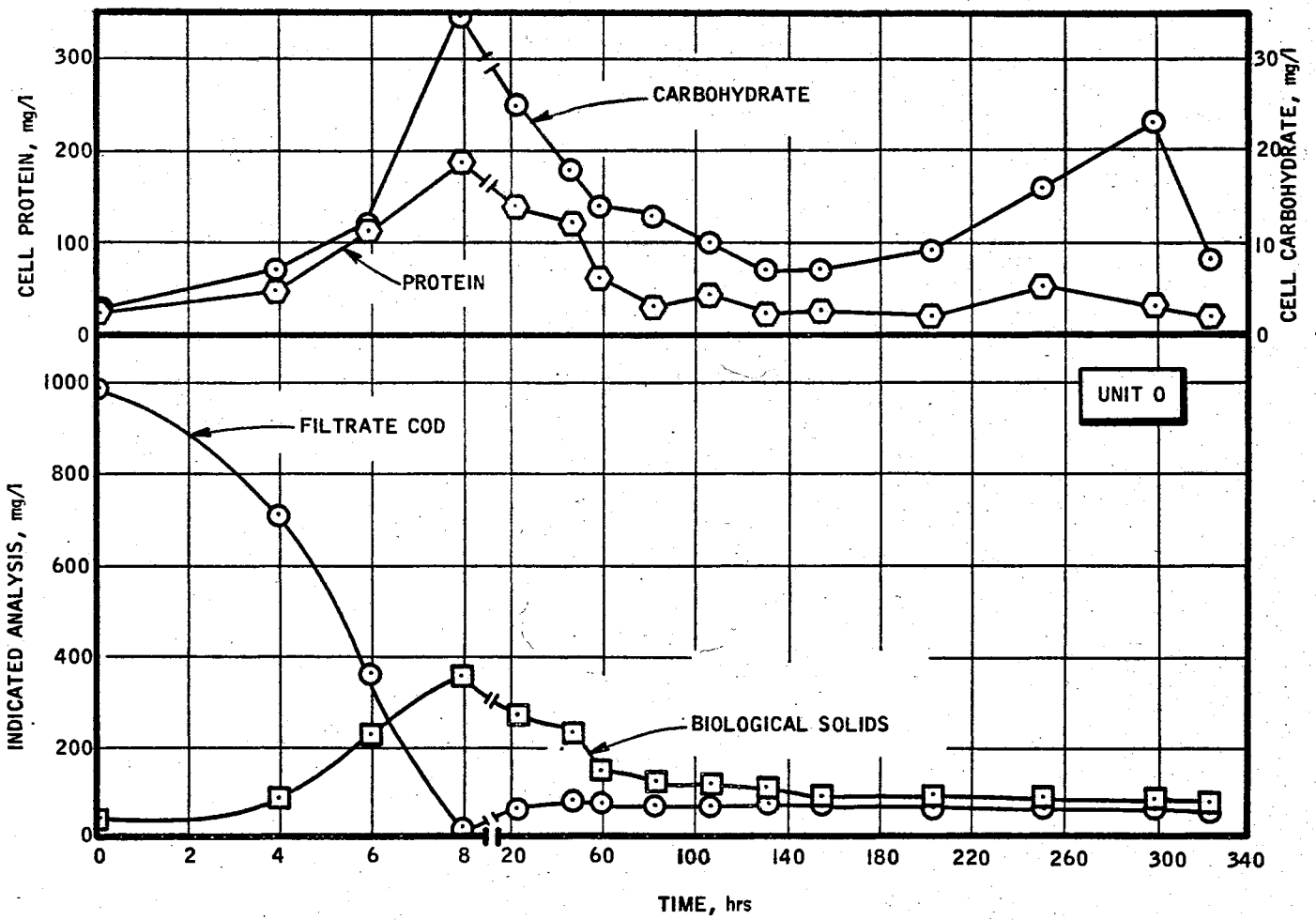

Figure 13. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 13.

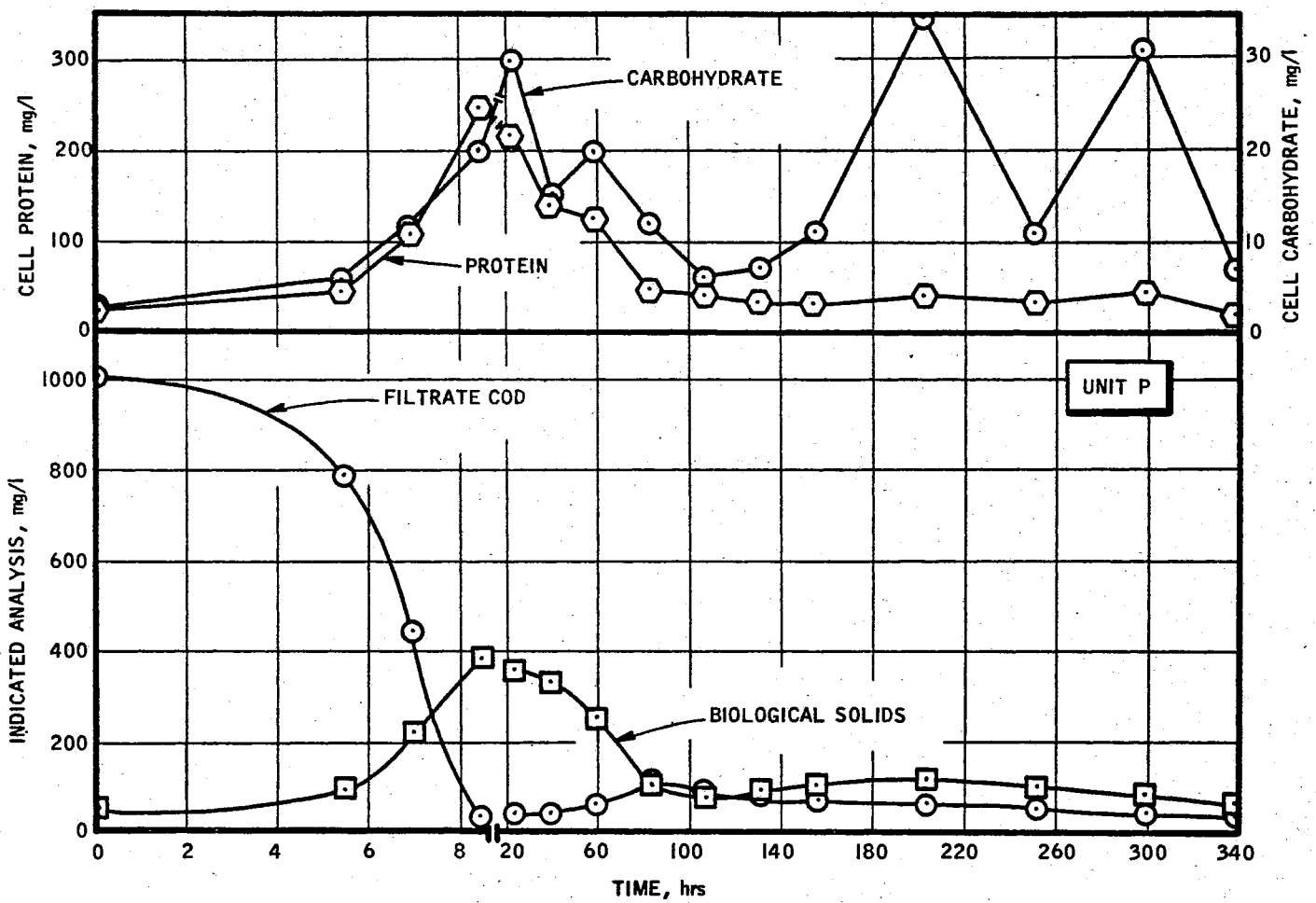

Figure 14. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 14.

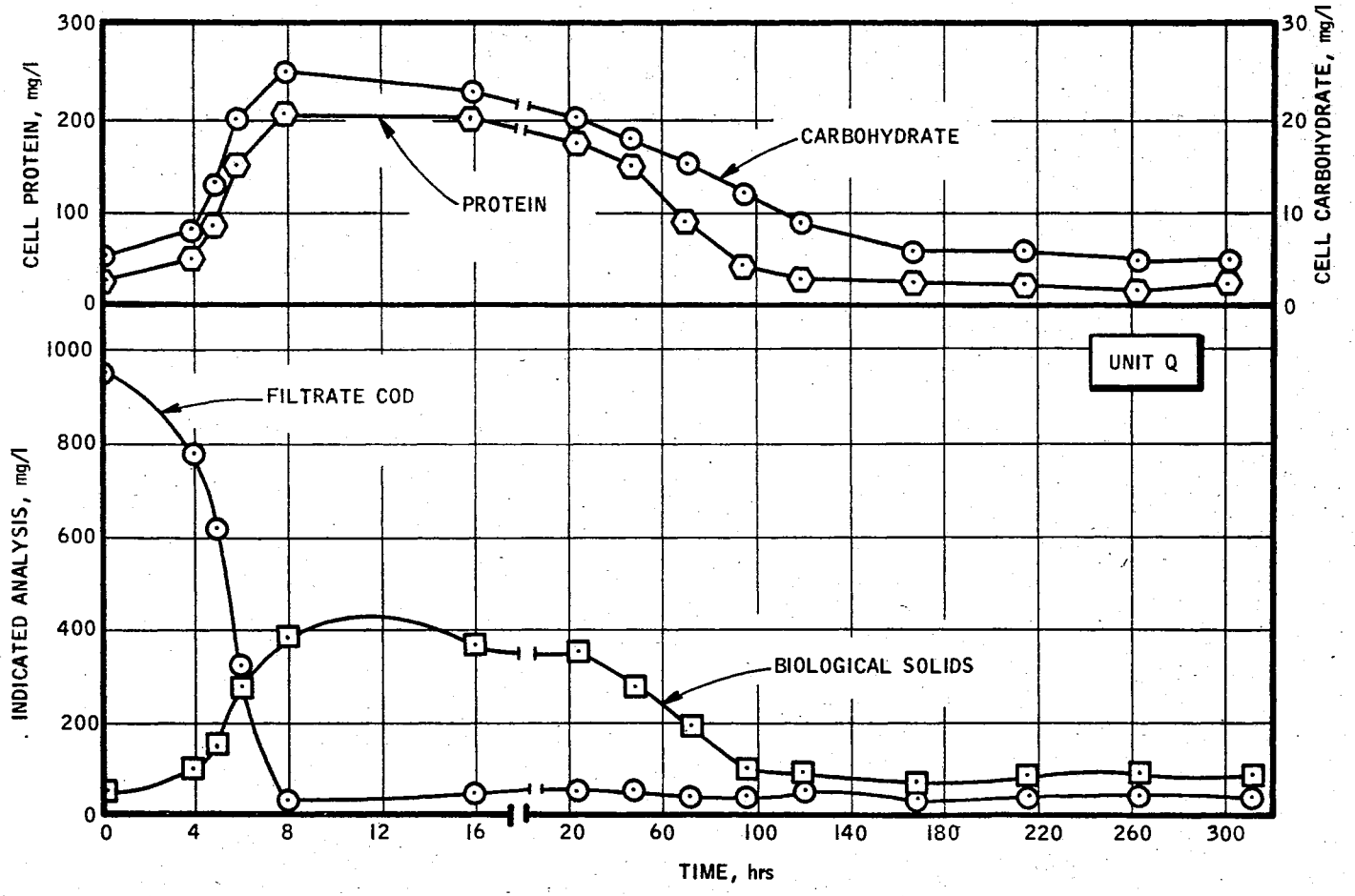

Figure 15. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 15.

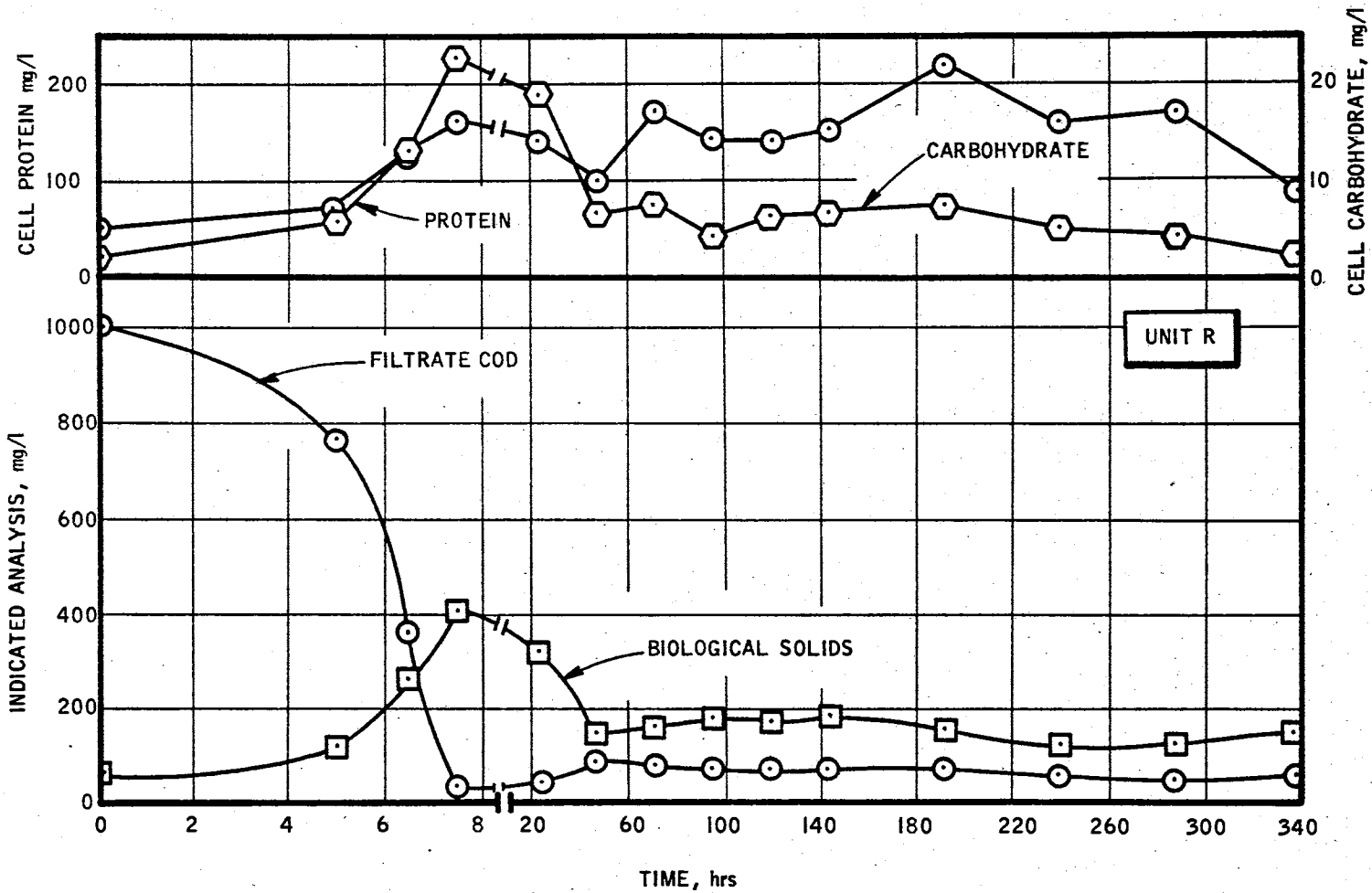

Figure 16. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 16.

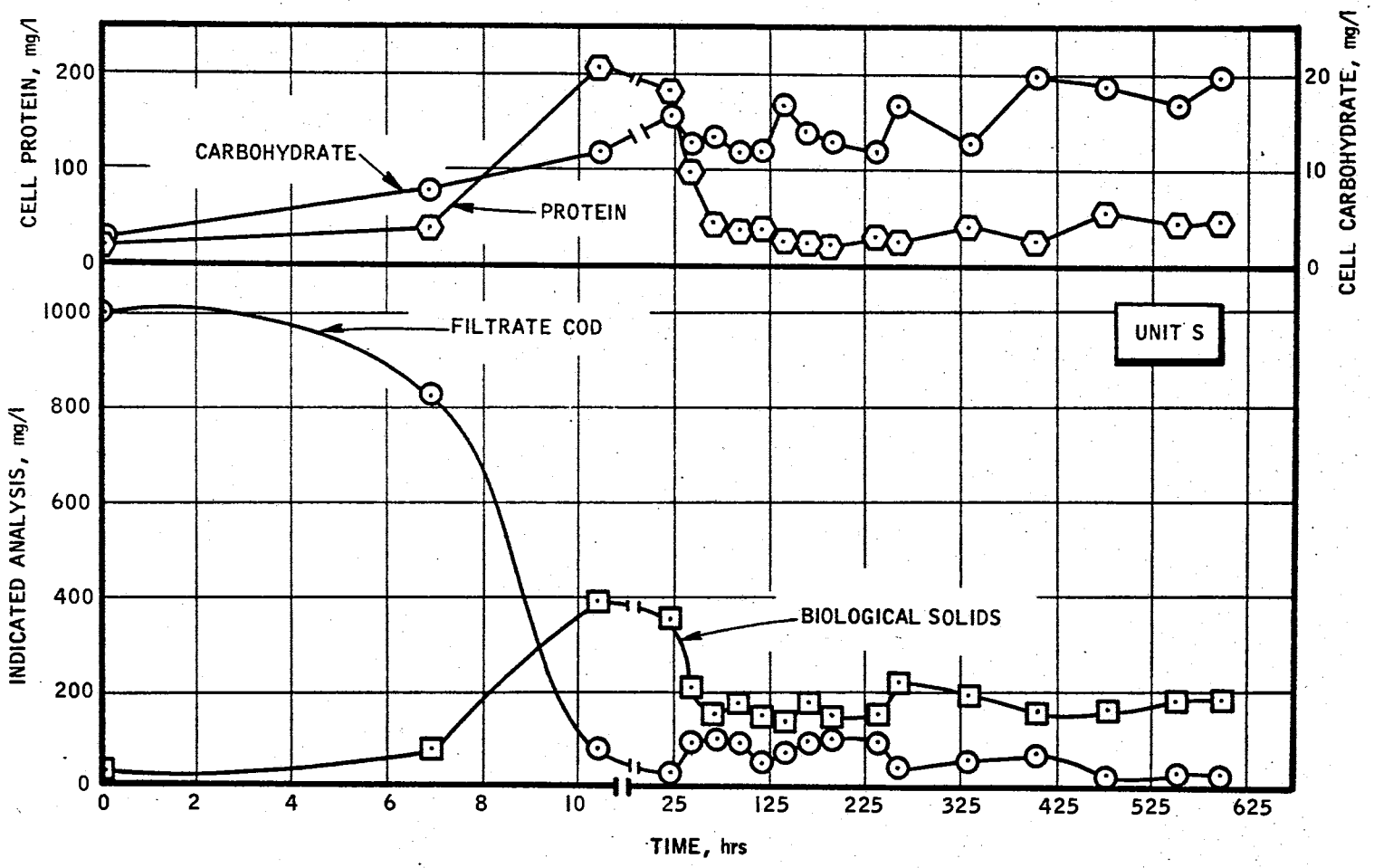

Figure 17. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 17.

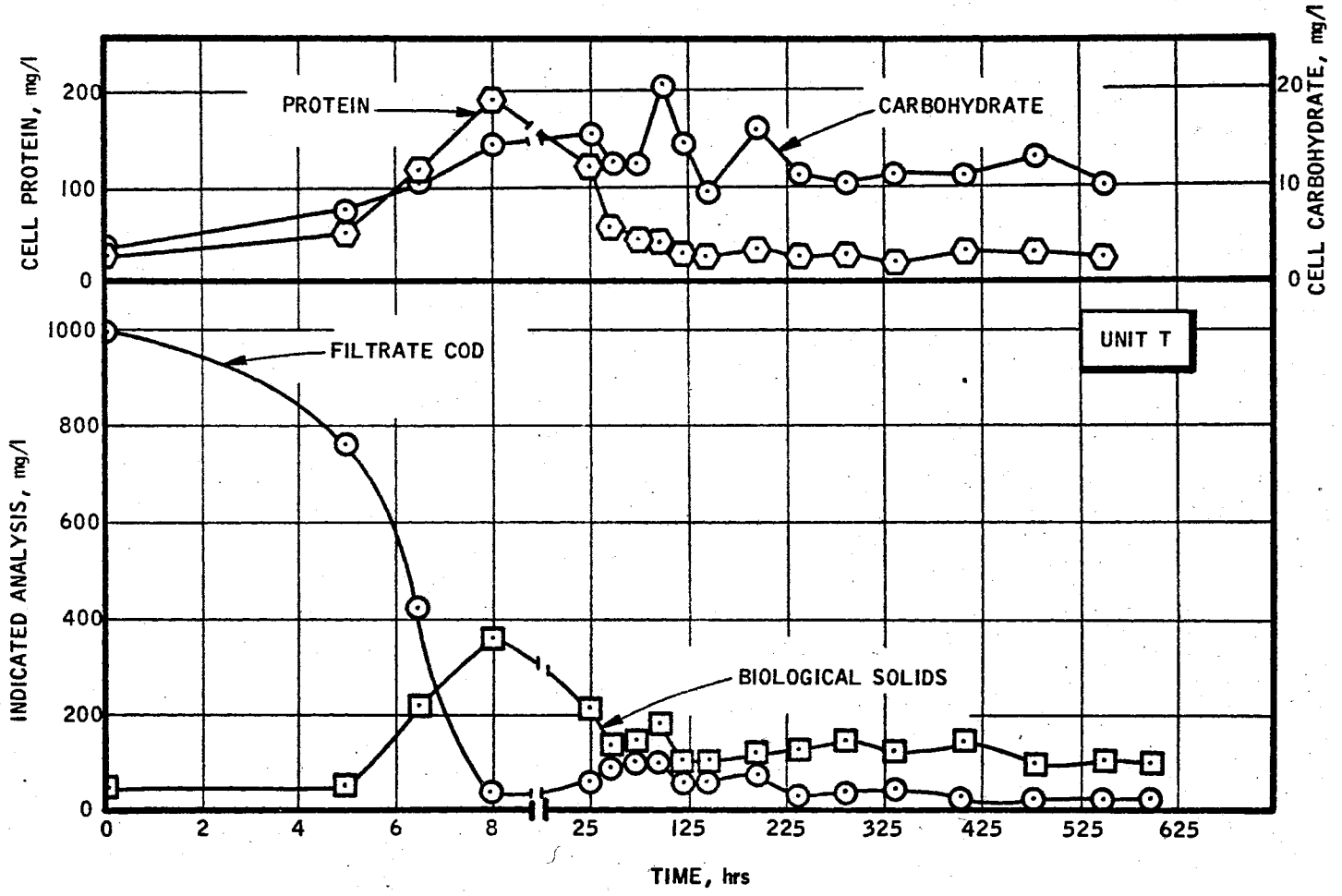

Figure 18. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 18.

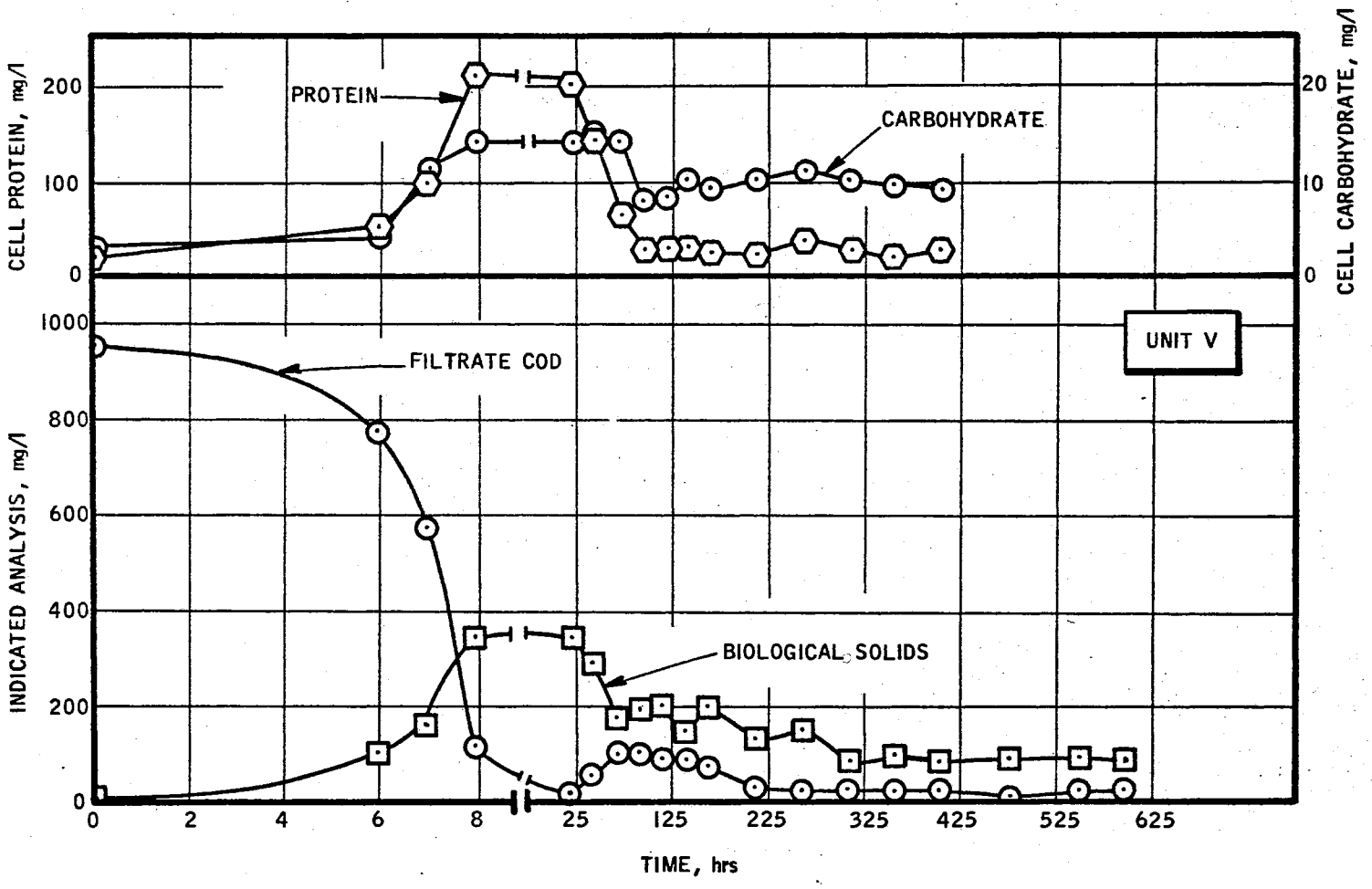

Figure 19. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 19.

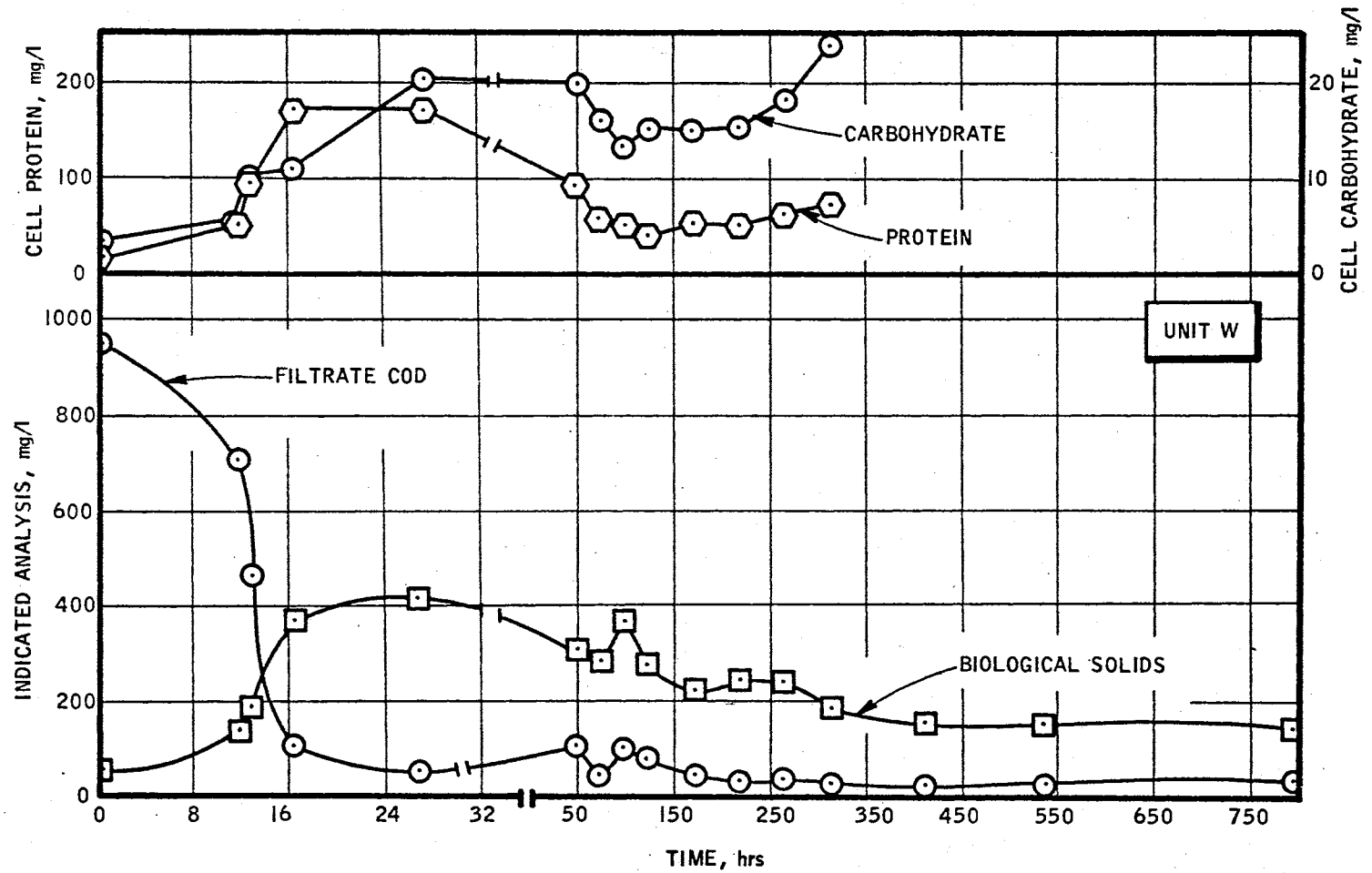

Figure 20. Metabolic response of an acclimated natural microbial population growing on acetic acid - Experiment No. 20.

experiments 11-20 (Figures 11-20). Table III is a summary of characteristics of acetic acid systems 8-20 at the time of removal of exogenous substrate.

B. Growth Kinetics

1. Cell Yield

Figures 21-40 (experiments 1-20) show sludge yield values obtained by determining the slope of a plot of biological solids vs. substrate (COD) removed. On each figure, the end of the logarithmic growth phase of the activated sludge mixed microbial population is marked. This point was located by generating a growth curve (a plot of optical density [log] vs. time) and determining the time at which the logarithmic grown phase for each experiment (Figures 1-20) started to end and the declining growth phase began. For Figures 28-34, and 36-40, data points obtained by correlation of optical density, solids growth, and substrate removal curves are denoted by a single circle.

It can be seen from Figures 21-40 (experiments 1-20) that sludge yield during the entire period of autocatalytic growth of heterogeneous microbial populations metabolizing acetic acid can be considered constant. Seventy percent of the experiments (Figures 21-40) show the end of the logarithmic growth phase occurring after at least two-thirds of the substrate had been oxidized or assimilated. Results of statistical analysis of the cell yield values are shown in Table IV. Cell yield values for experiments 1-20 (Figures 1-20) are listed in Tables II and III and are in agreement with values reported in the literature (19)(12).

TABLE III

SUMMARY OF CHARACTERISTICS OF ACETIC ACID SYSTEMS 8-20 AT TIME OF REMOVAL OF EXOGENOUS SUBSTRATE

			COD		Biochemical Efficiency	Sludge Synthesis			Sludge Carbohydrate Total		Sludge Protein Total		Physiological Growth Constants	
Exper. No.	Fig. No.	Time (hrs.)	Residual mg/l	Removed mg/l	%	Total mg/l	mg/l	Yield Percent	mg/l	As % Sludge Dry Wt.	mg/l	As % Sludge Dry Wt.	μ_{max} hr^{-1}	K_s mg/l
8	8	8.5	44	866	95	378	333	43.5	18	4.7	217	57.4	0.455	70
9	9	7.5	40	860	95	338	275	41.7	19	5.7	150	44.3		
10	10	7.5	28	912	97	330	260	43.3	14	4.2	170	51.5	0.518	33
11	11	7.0	20	940	98	387	362	45.7	20	5.1	173	44.7	0.715	45
12	12	7.0	8	972	99	357	332	41.2	17	4.7	167	46.7		
13	13	8.0	8	977	99	358	318	37.5	35	9.7	179	50.0	0.690	39
14	14	8.5	32	968	97	387	339	37.5	20	5.1	246	63.5	0.770	140
15	15	8.0	28	922	97	387	337	42.0	25	6.4	205	52.9		
16	16	7.5	36	964	97	403	340	38.5	16	3.9	229	56.8	0.770	36
17	17	10.5	8	920	99	393	373	39.5	12	3.0	208	52.9	0.685	34
18	18	8.0	36	964	97	365	320	35.0	14	3.8	184	50.4	0.690	94
19	19	8.0	116	849	80	343	328	40.8	14	4.0	212	61.8	0.775	29
20	20	27.0	56	894	94	413	355	41.0	20	4.8	170	41.1	0.706	62

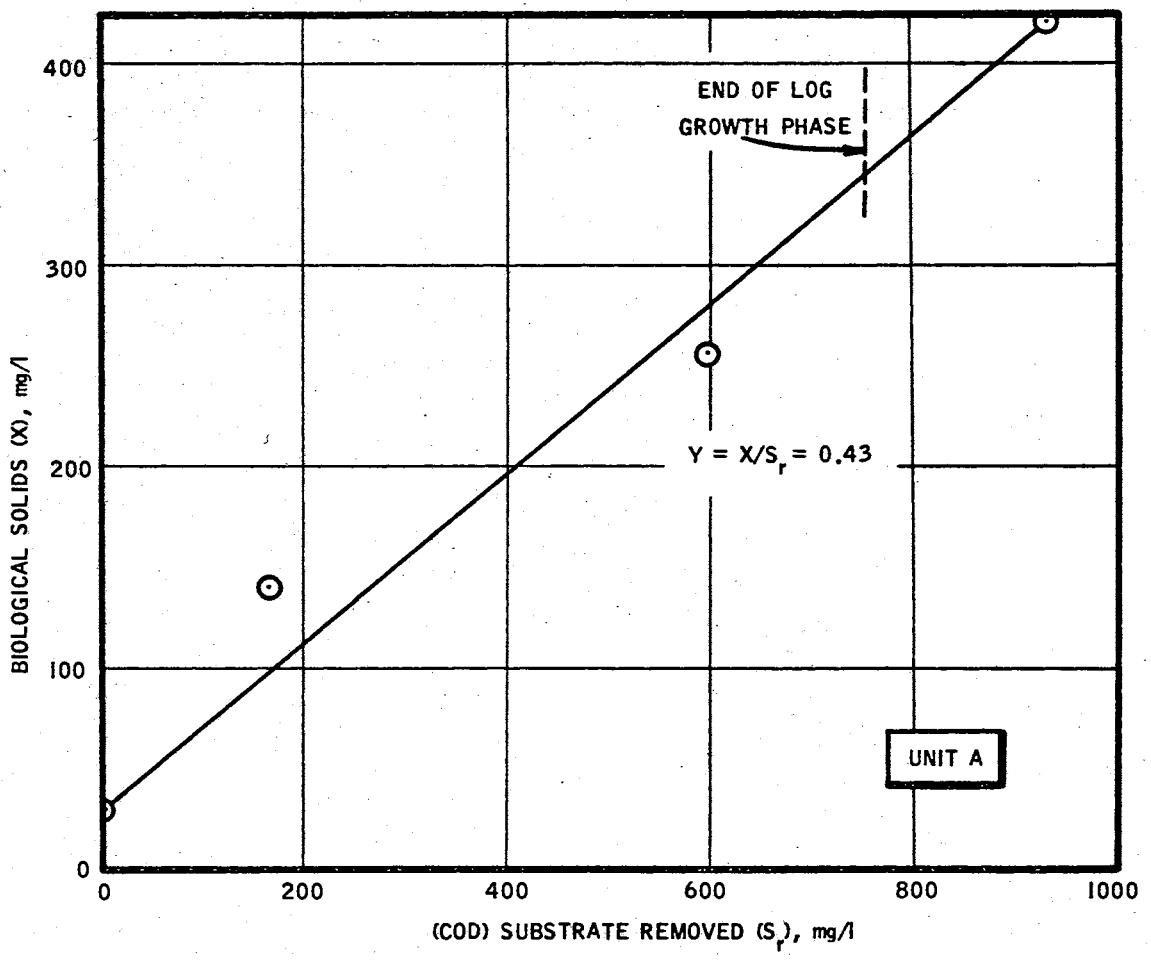

Figure 21. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 1.

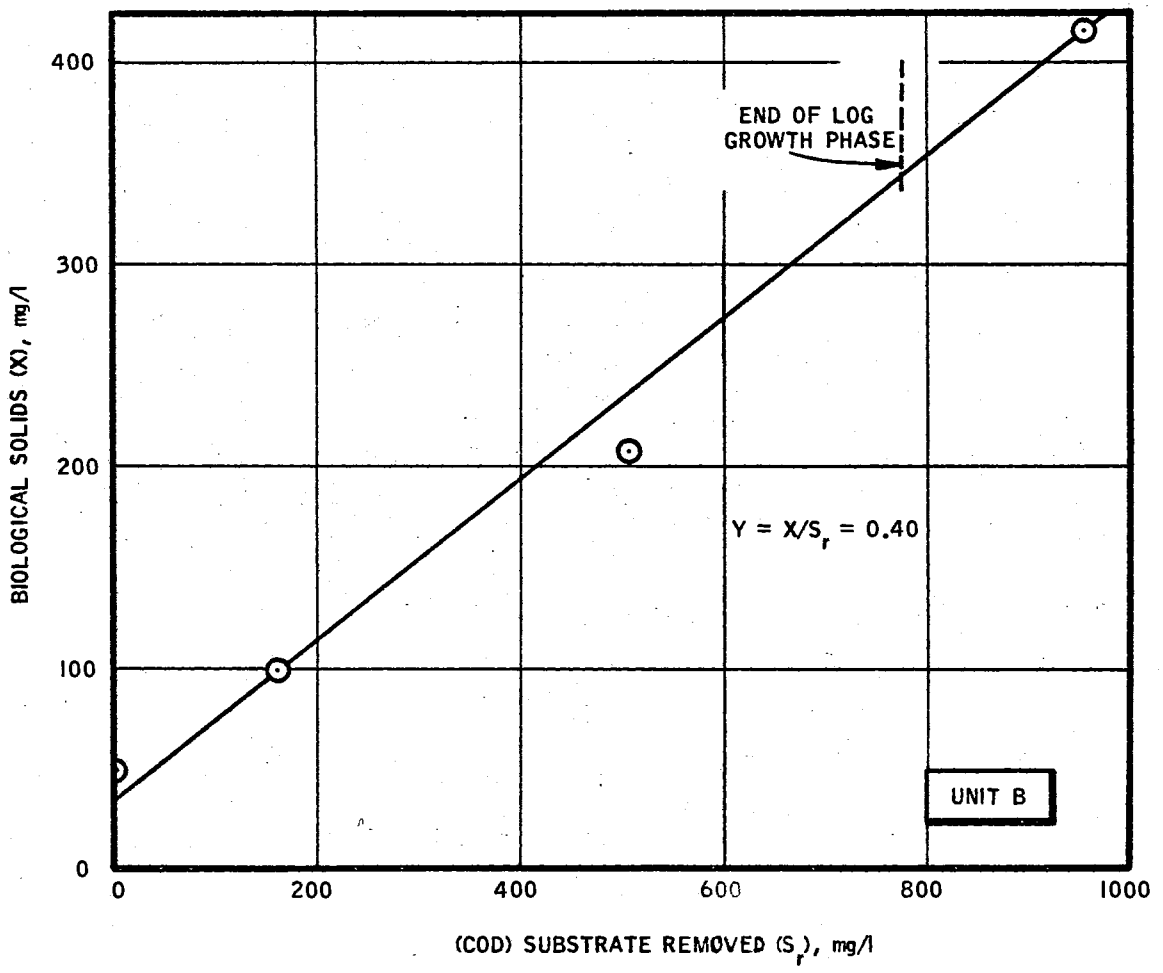

Figure 22. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 2.

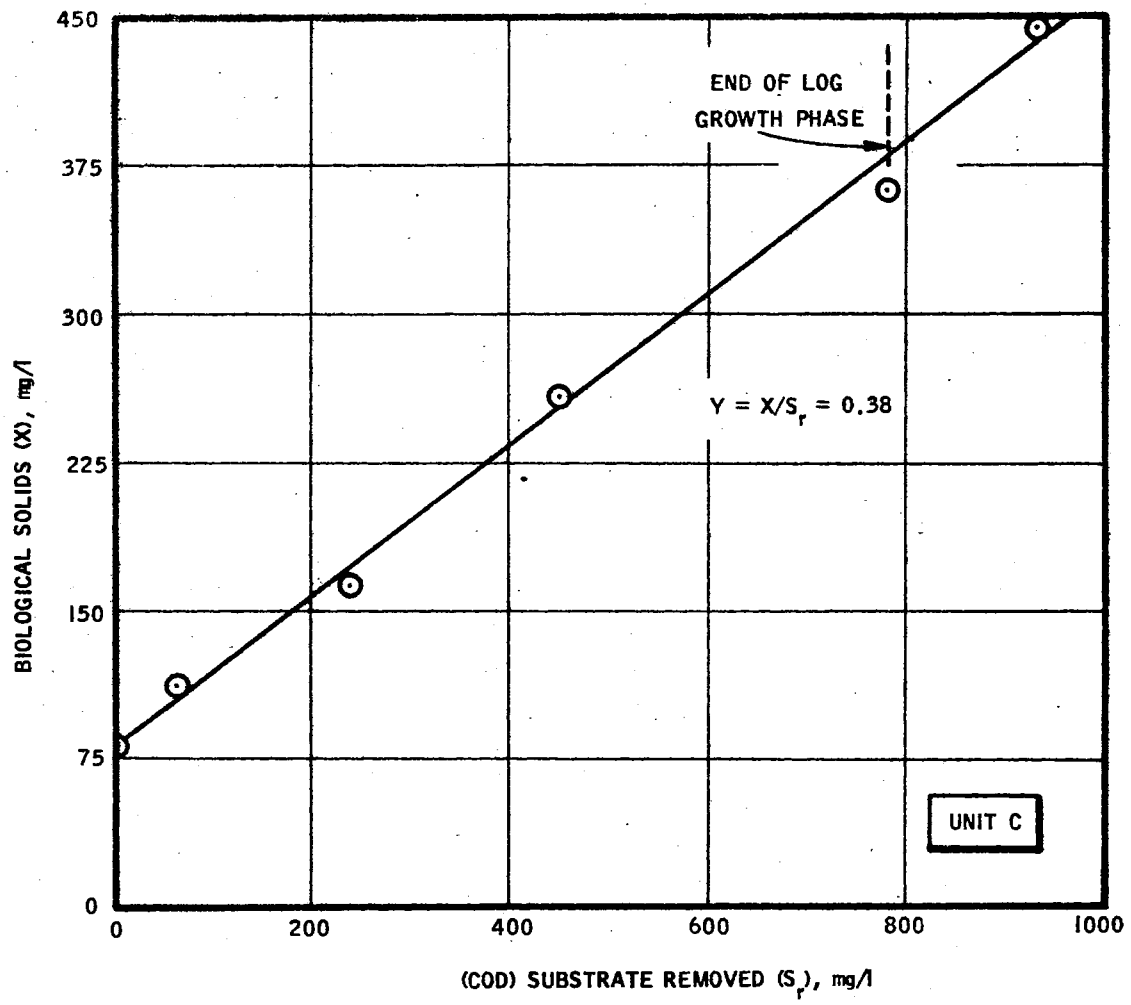

Figure 23. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 3.

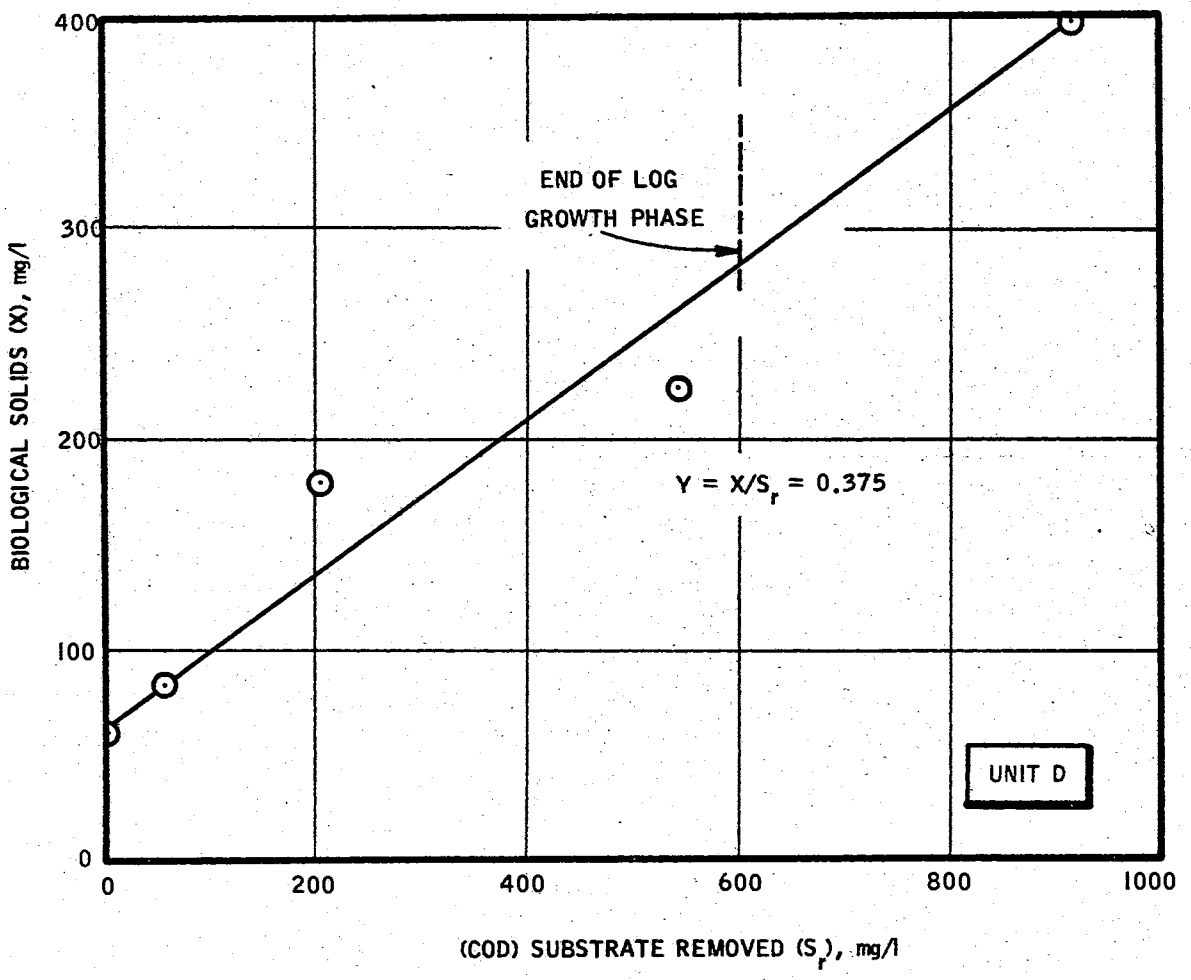

Figure 24. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 4.

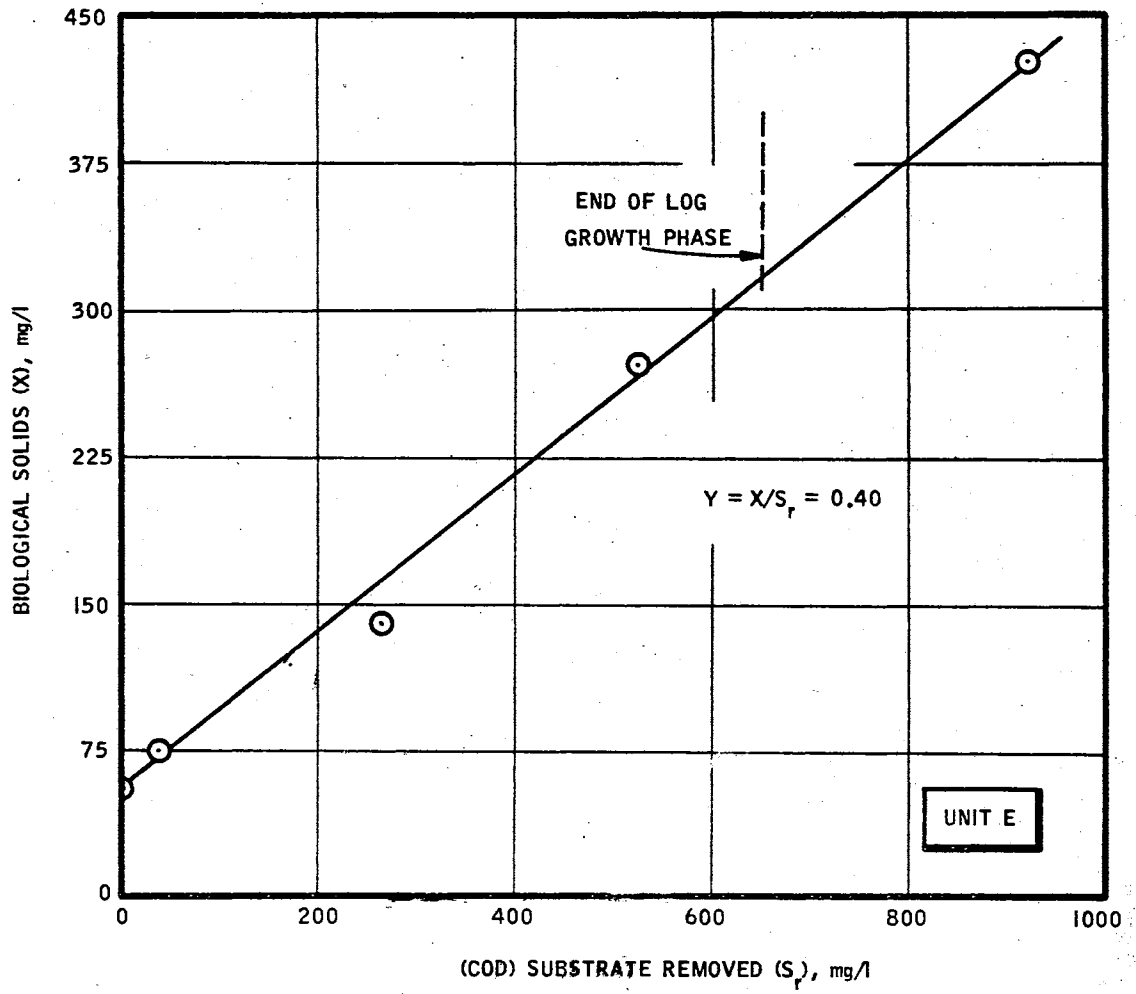

Figure 25. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 5.

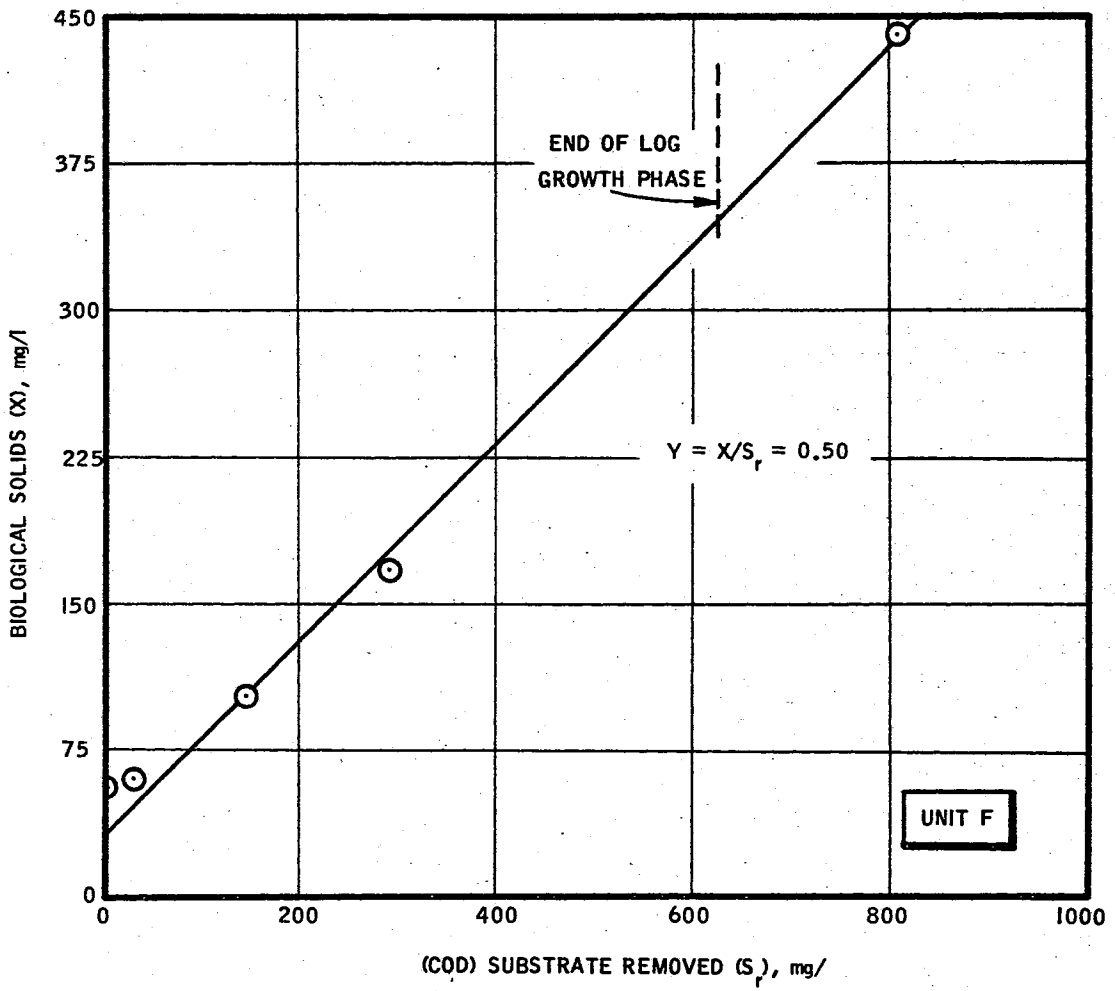

Figure 26. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 6.

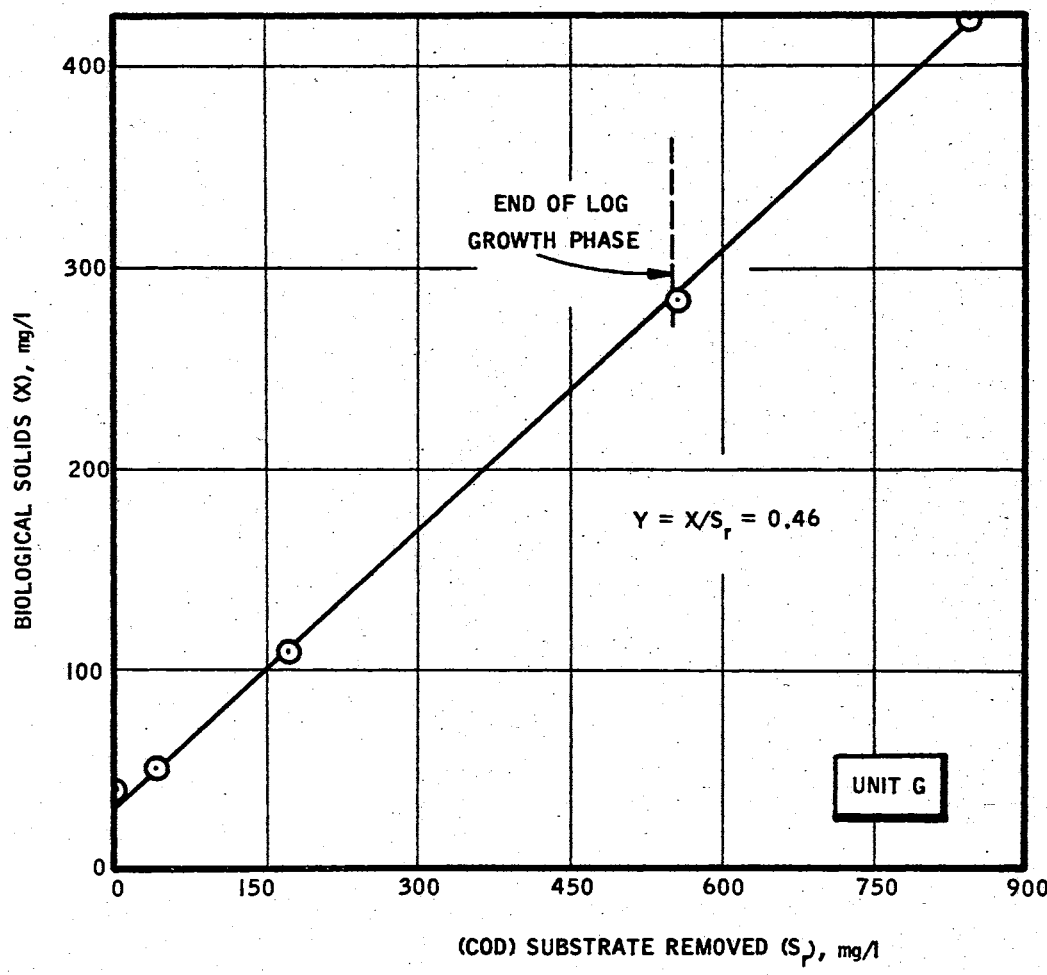

Figure 27. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 7.

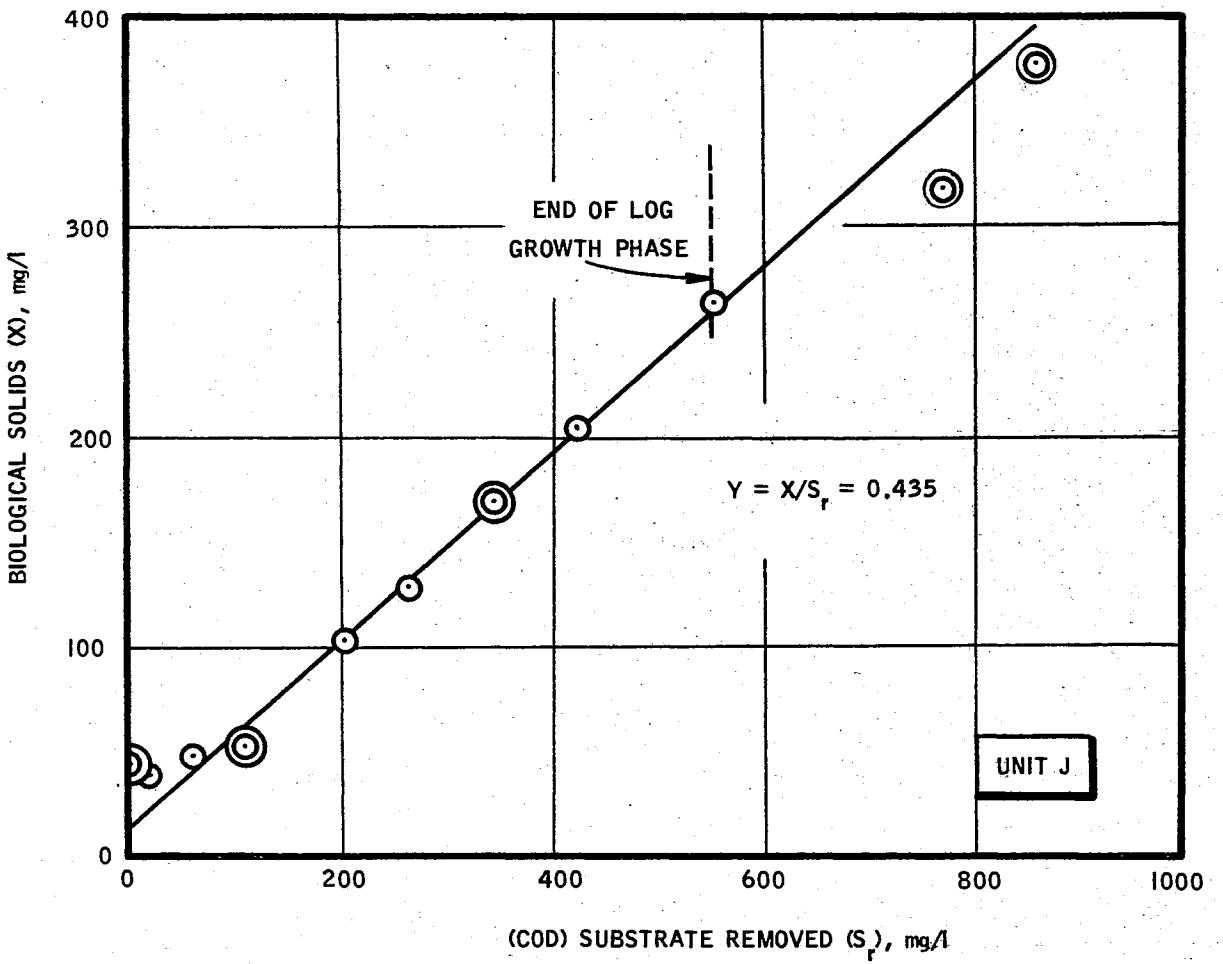

Figure 28. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 8.

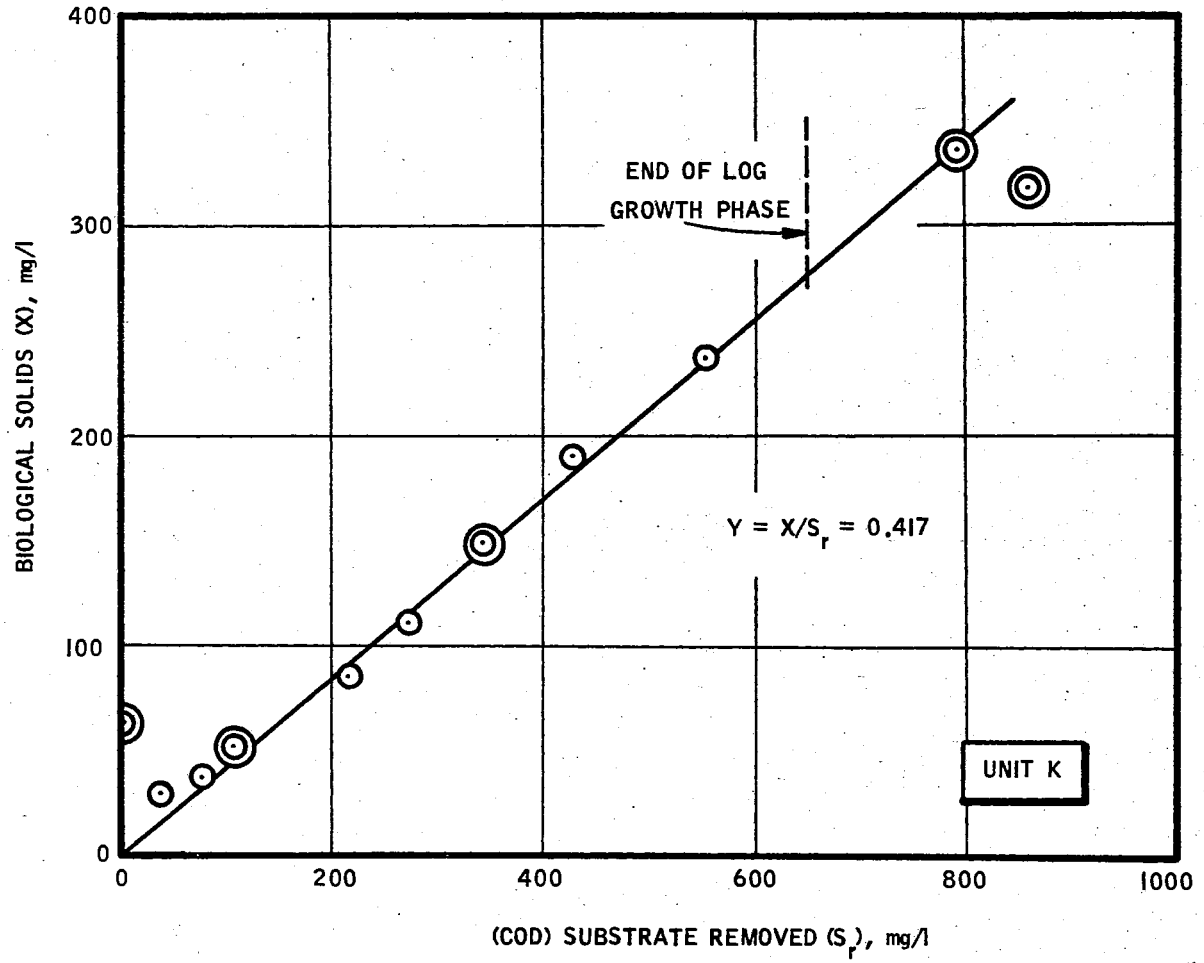

Figure 29. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 9.

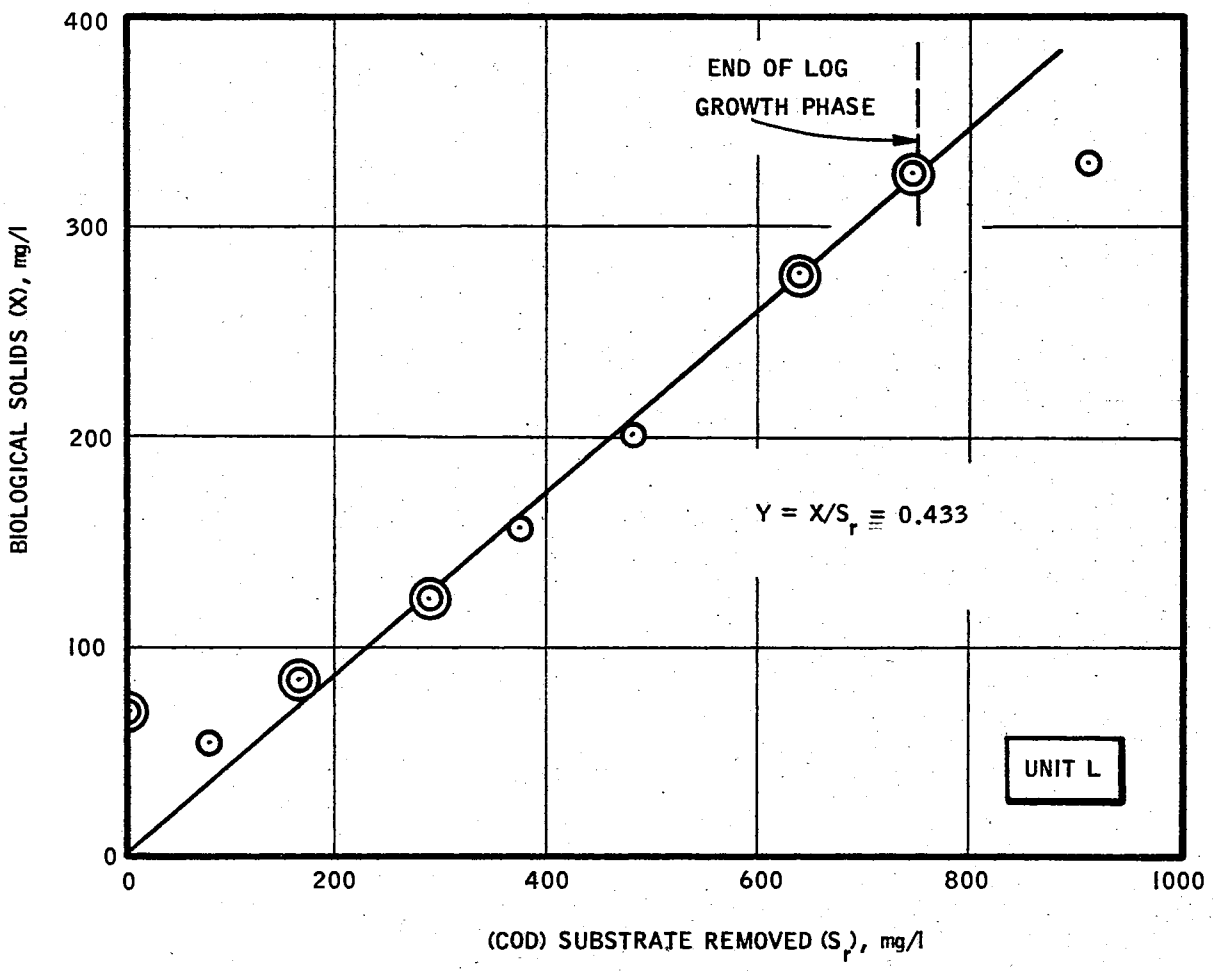

Figure 30. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 10.

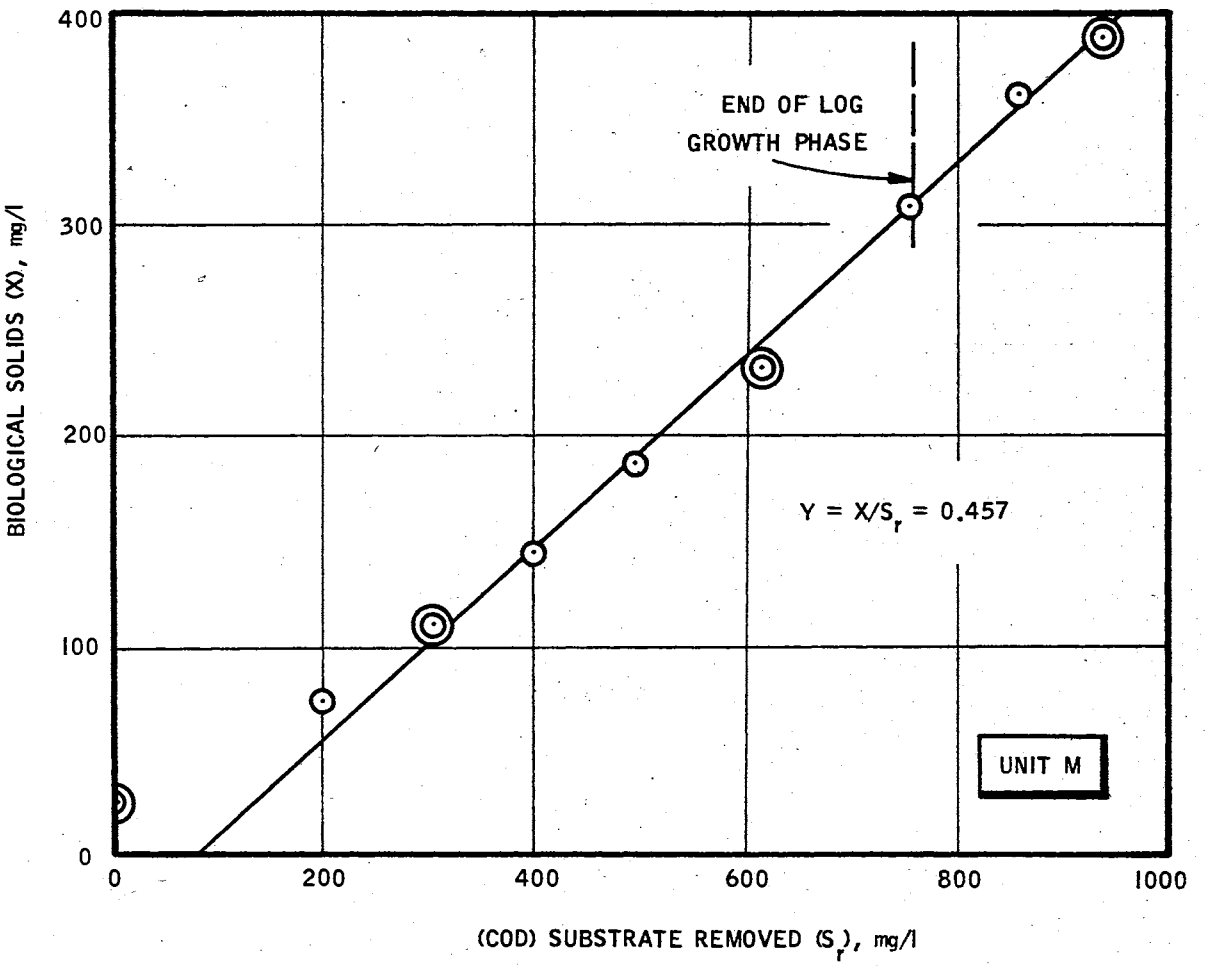

Figure 31. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 11.

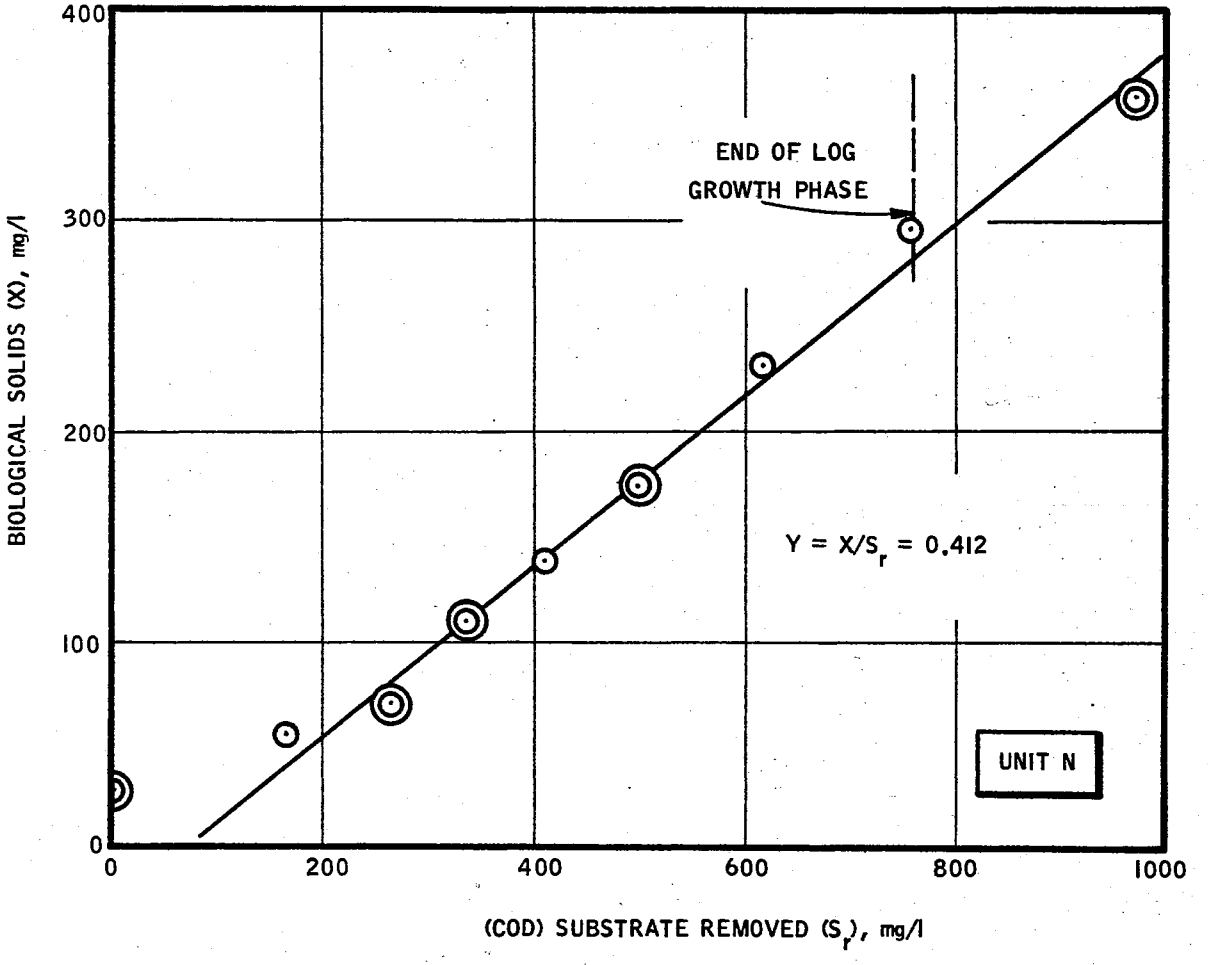

Figure 32. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 12.

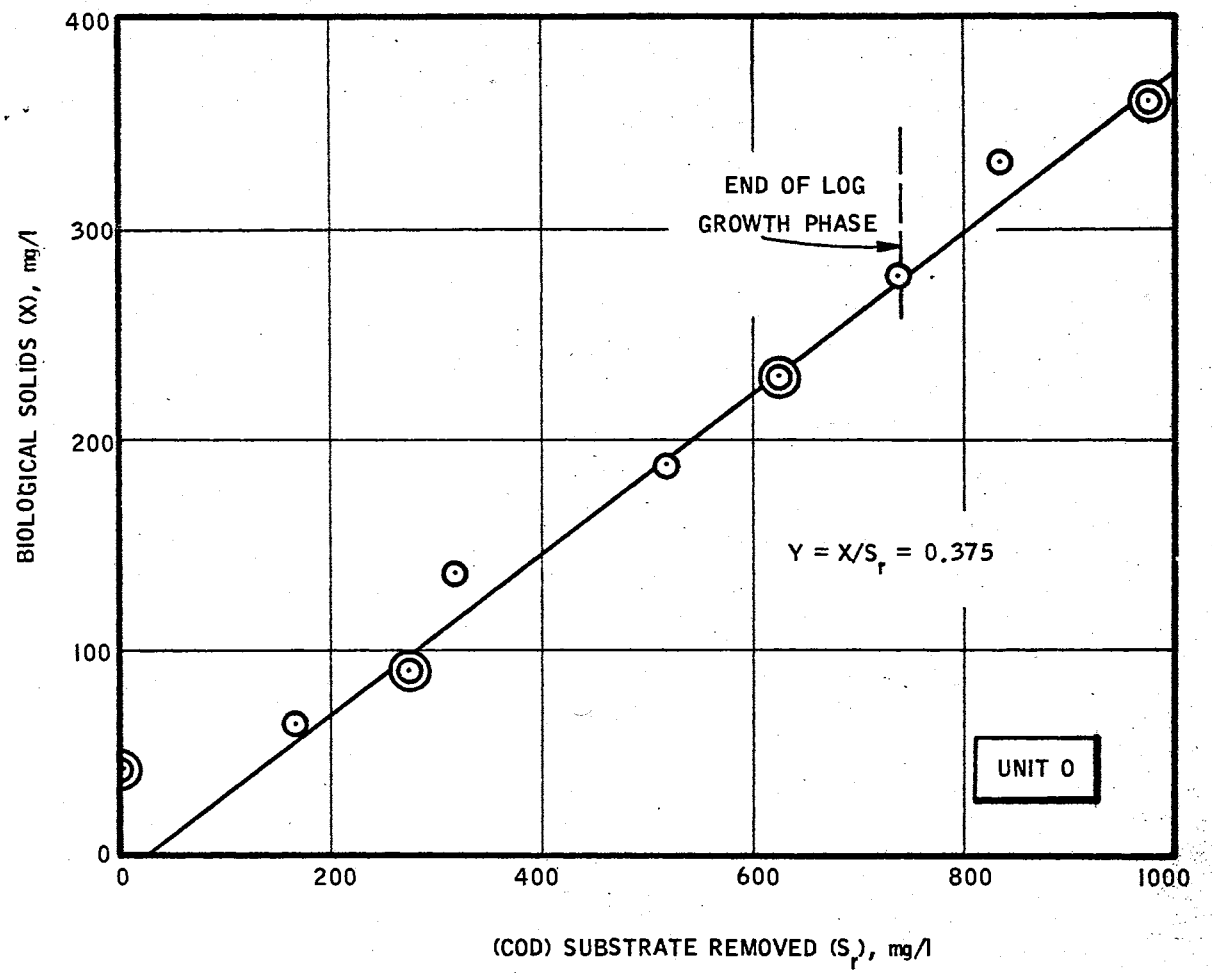

Figure 33. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 13.

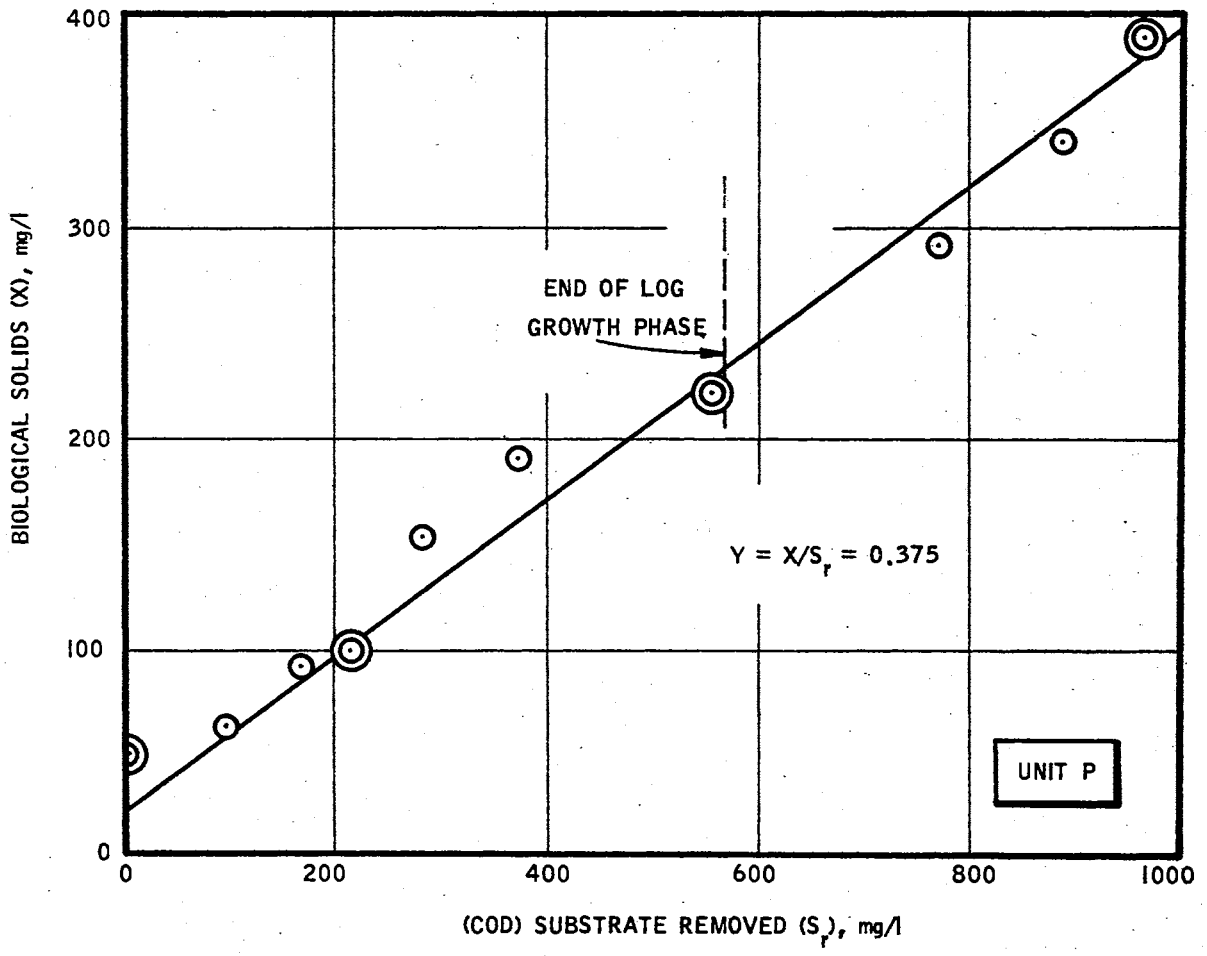

Figure 34. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 14.

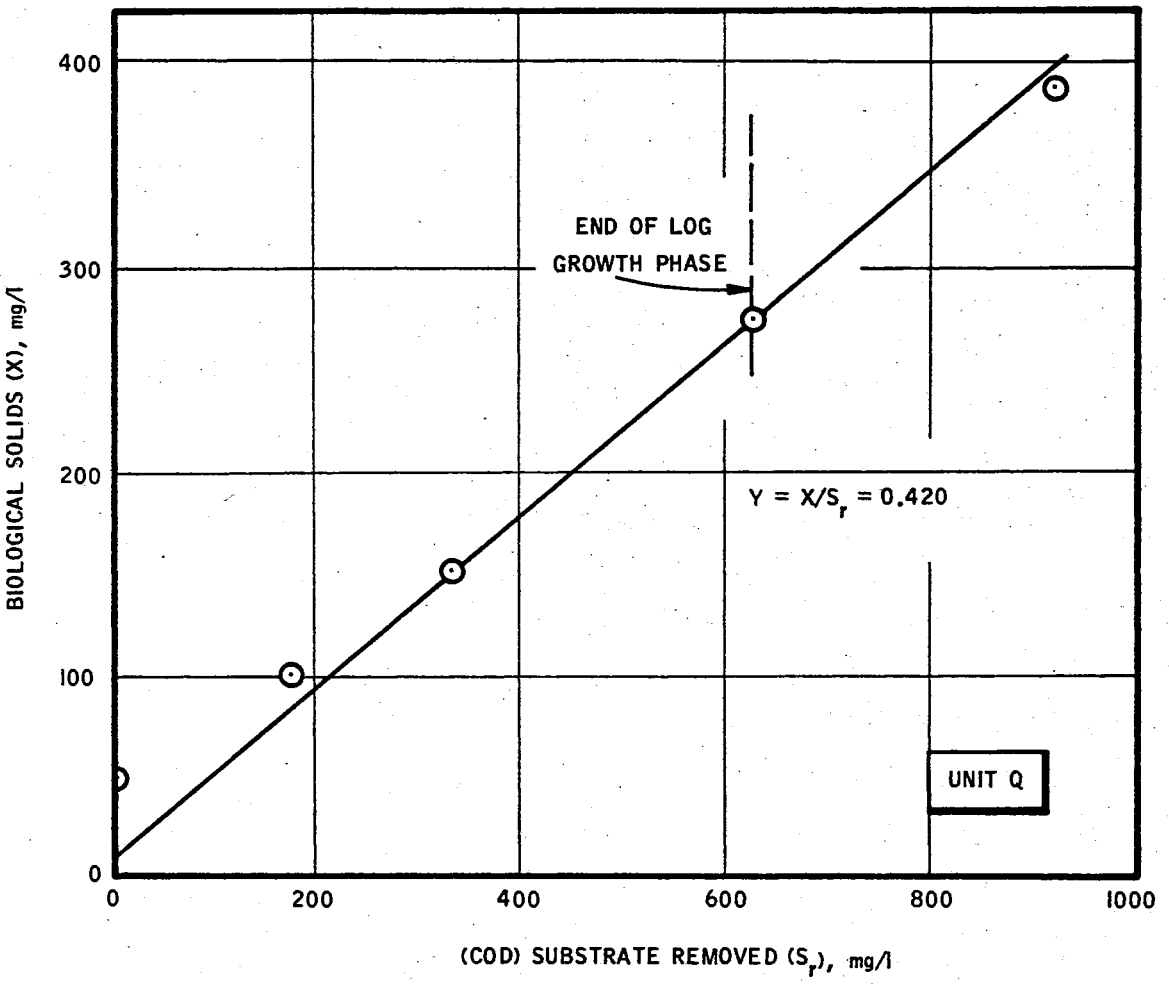

Figure 35. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 15.

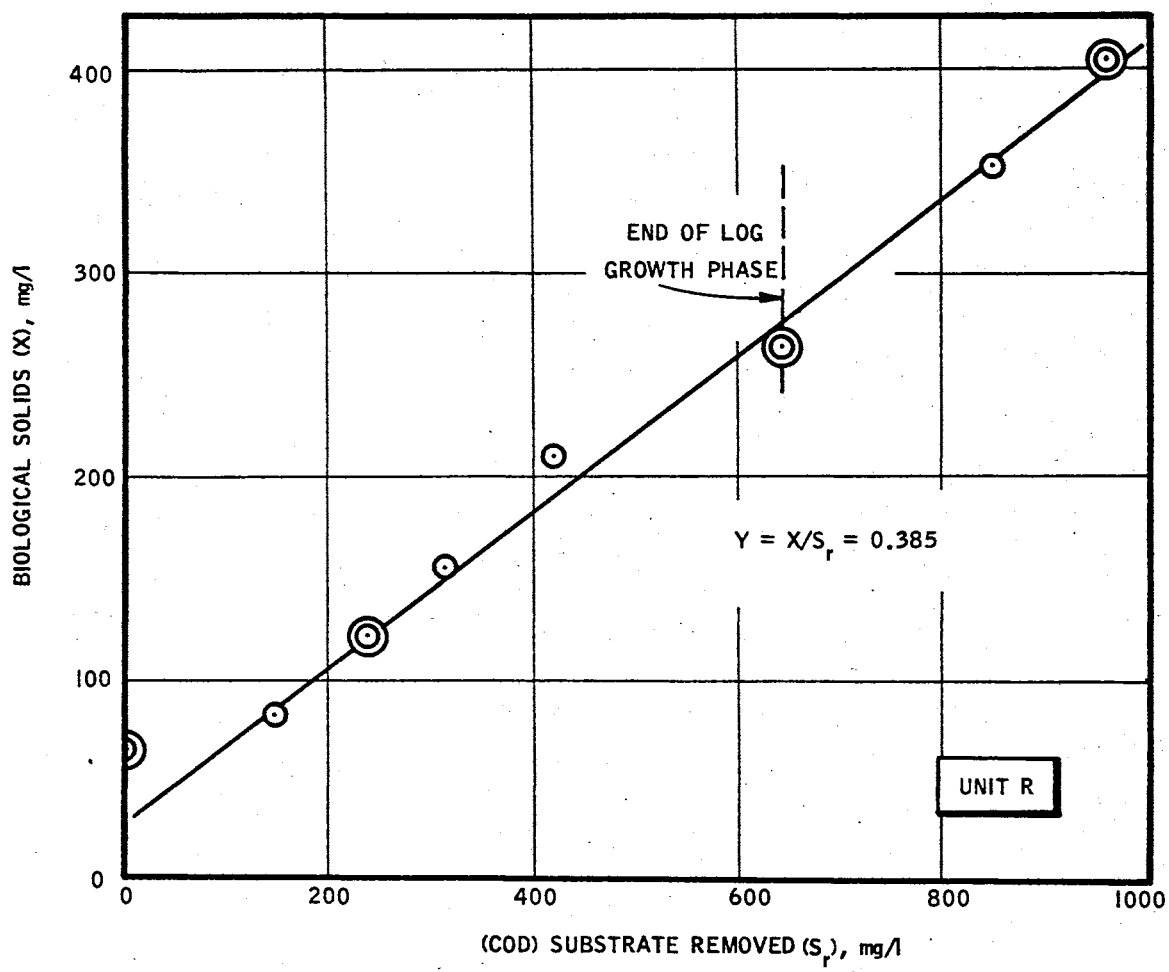

Figure 36. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 16.

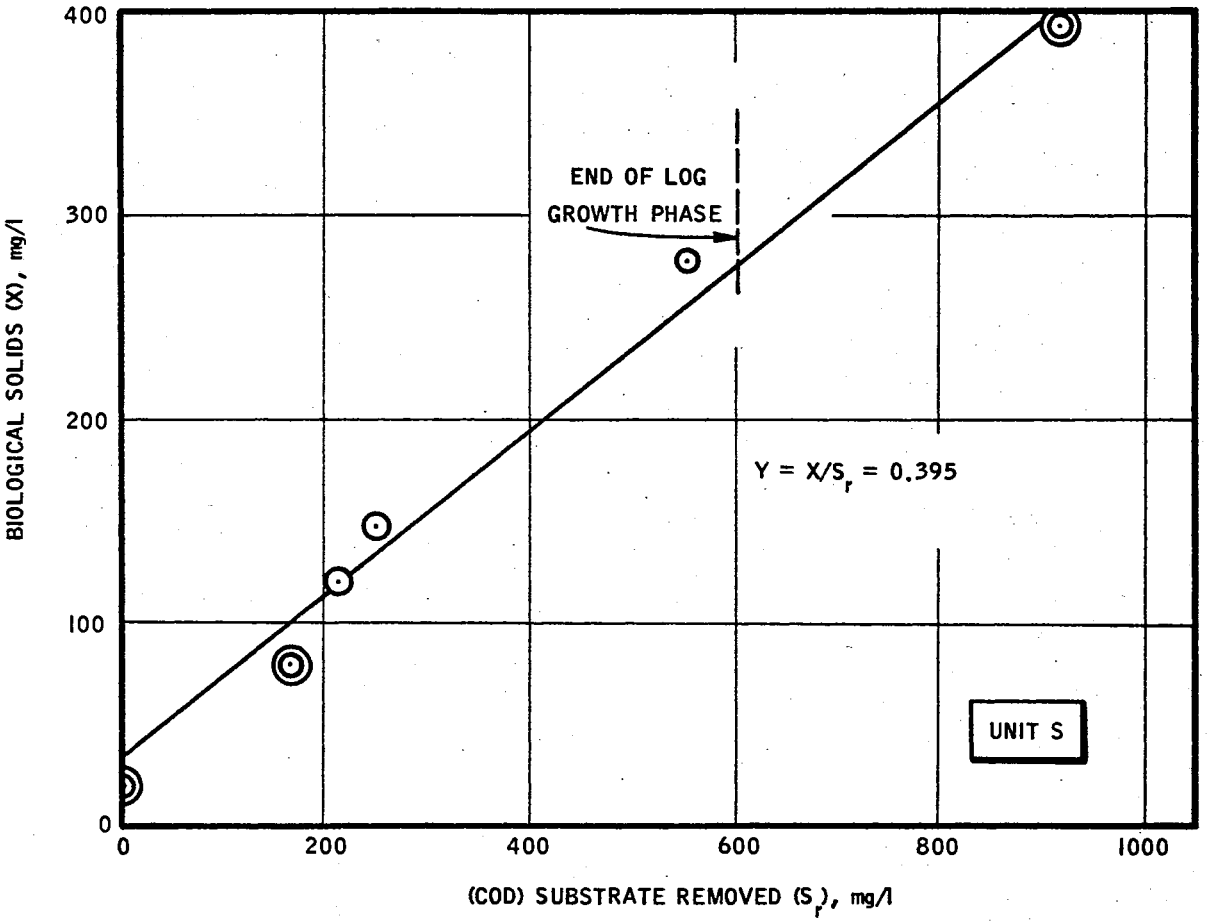

Figure 37. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 17.

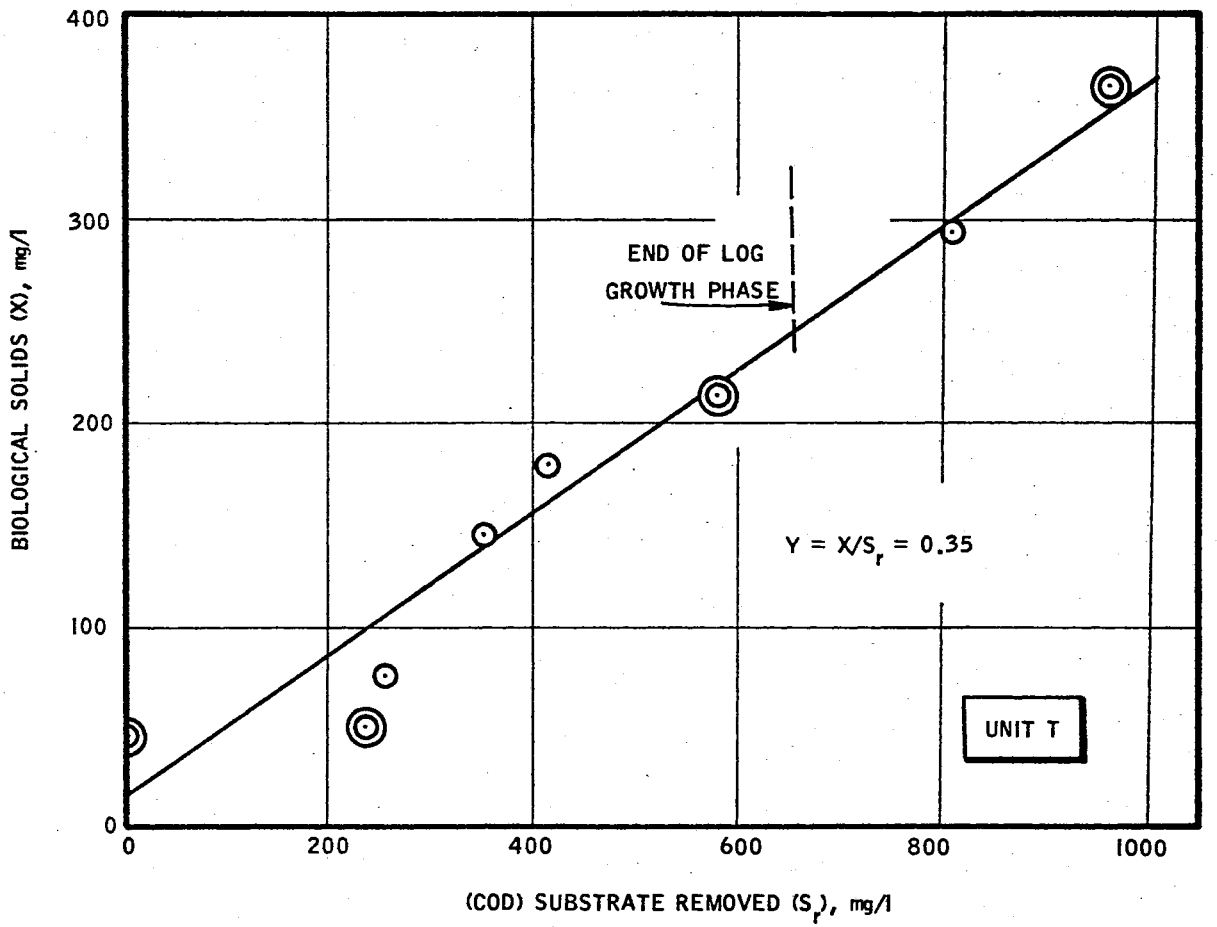

Figure 38. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 18.

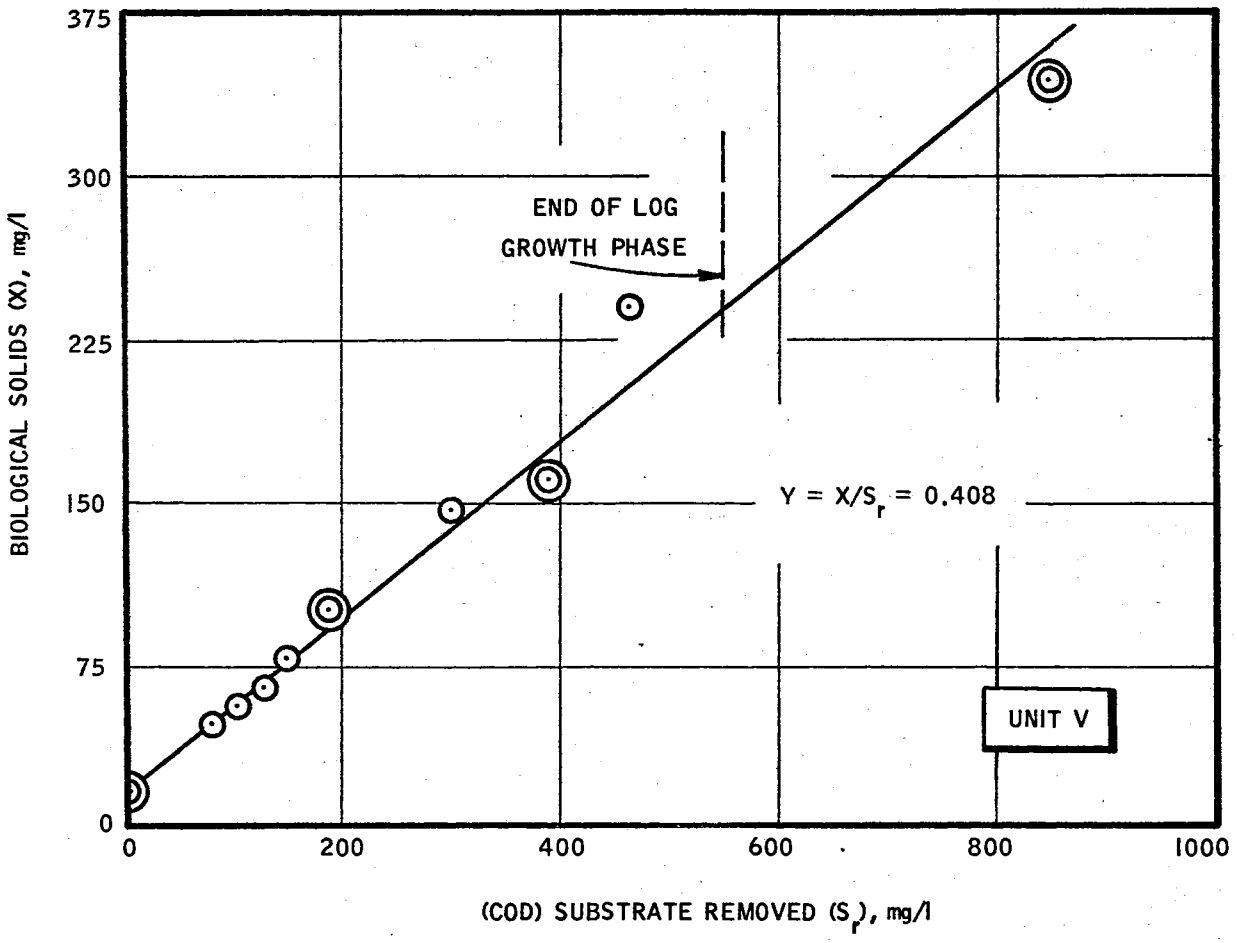

Figure 39. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 19.

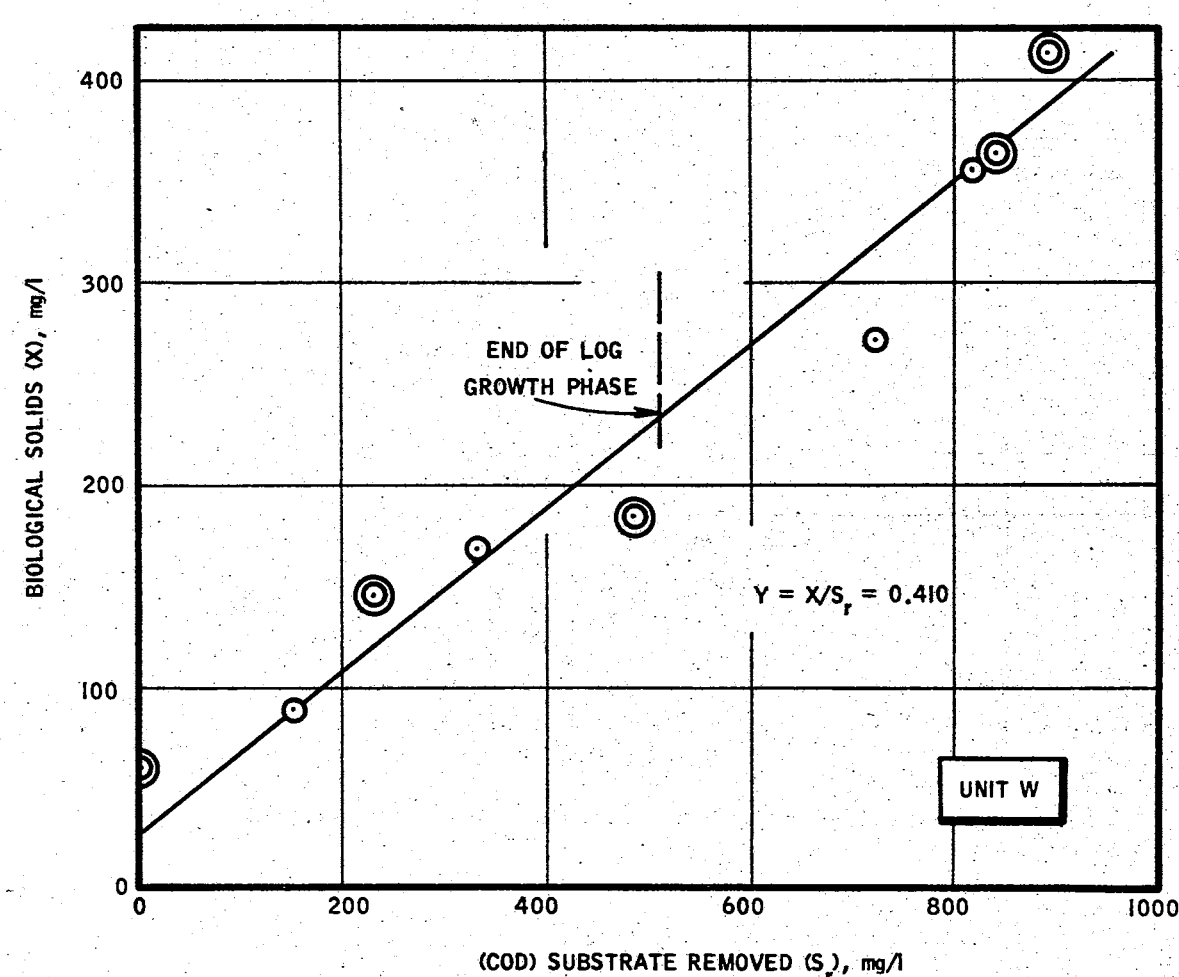

Figure 40. Cell yield for a heterogeneous microbial population growing on acetic acid - Experiment No. 20.

TABLE IV

STATISTICAL SUMMARY OF METABOLIC PARAMETERS

	n	Mean	Standard Deviation	Coefficient of Variance	95% CL
Cell yield	20	41.1	3.53	8.6	41.1 $\pm$ 1.57
μ_{max}	11	0.705	0.137	19.4	0.705 $\pm$ 0.090
K_s	11	60	33.7	56.2	60 $\pm$ 23.8

2. Growth Rate Experiments

Figures 41-69 show the effect of substrate concentration on exponential growth rate (μ). Graphical representation of exponential growth rate (μ) substrate concentration data by a rectangular hyperbola (Monod plot) and by a double reciprocal straight-line plot (Lineweaver, Burk) are presented in Figures 41-62. A representative example of optical density (log) vs. time plots for calculation of exponential growth rate (μ) is given in Figures 63-69 (experiment 11). The values for the physiological growth constants maximum specific growth rate (μ_{max}) and saturation constant (K_s) were determined from the Lineweaver, Burk plots, and ranged from 0.455 to 0.980 hr^{-1} and 29 to 140 mg/l, respectively.

It can be seen from Figures 41-62 that the relationship, a single function of decreasing slope, proposed by Monod, fits the bulk of the data of μ and S reported herein. However, in contrast to Monod's theory that exponential growth can occur only in the presence of excess substrate, Figures 63-69 indicate exponential growth (as determined by

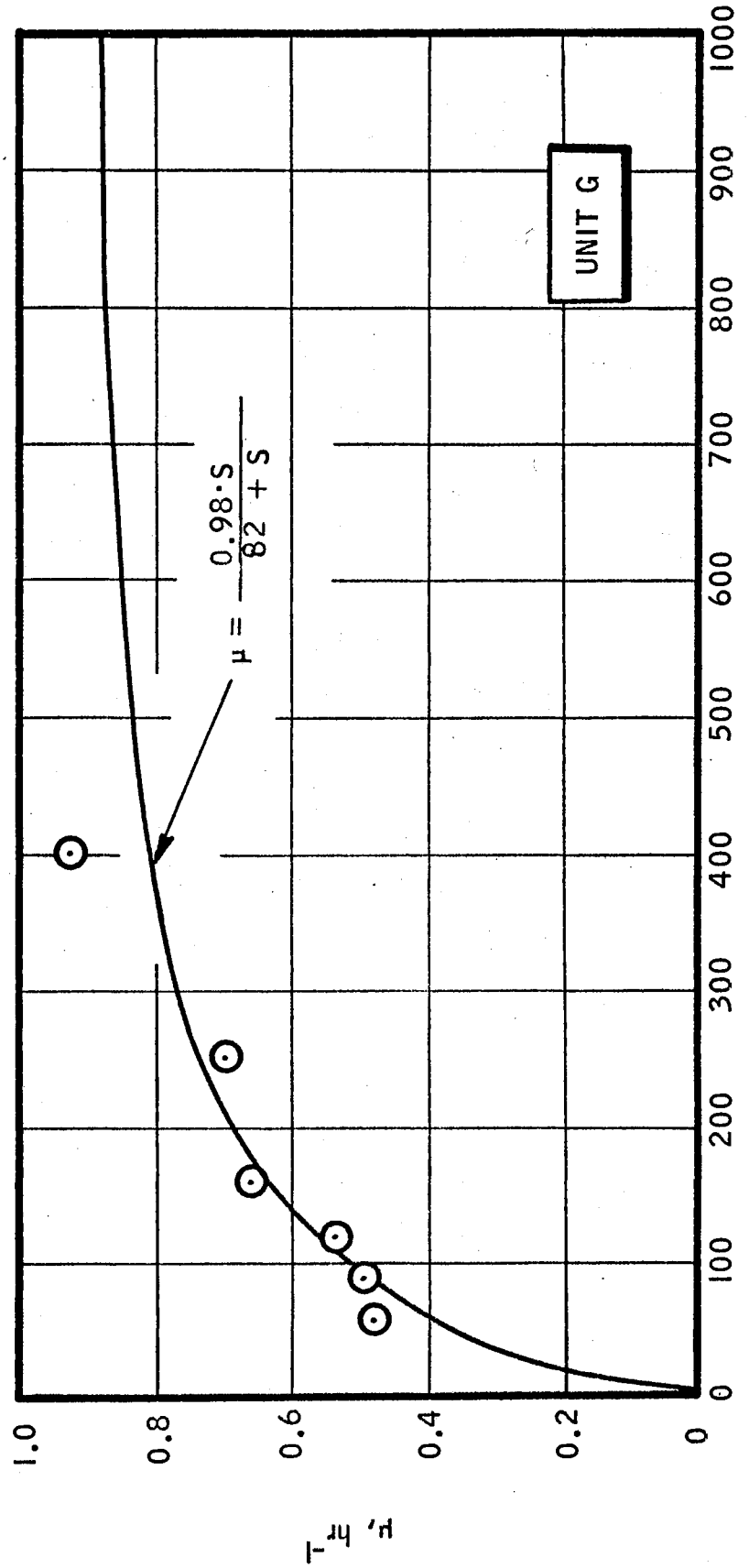

Figure 41. "Monod" plot for Experiment No. 7.

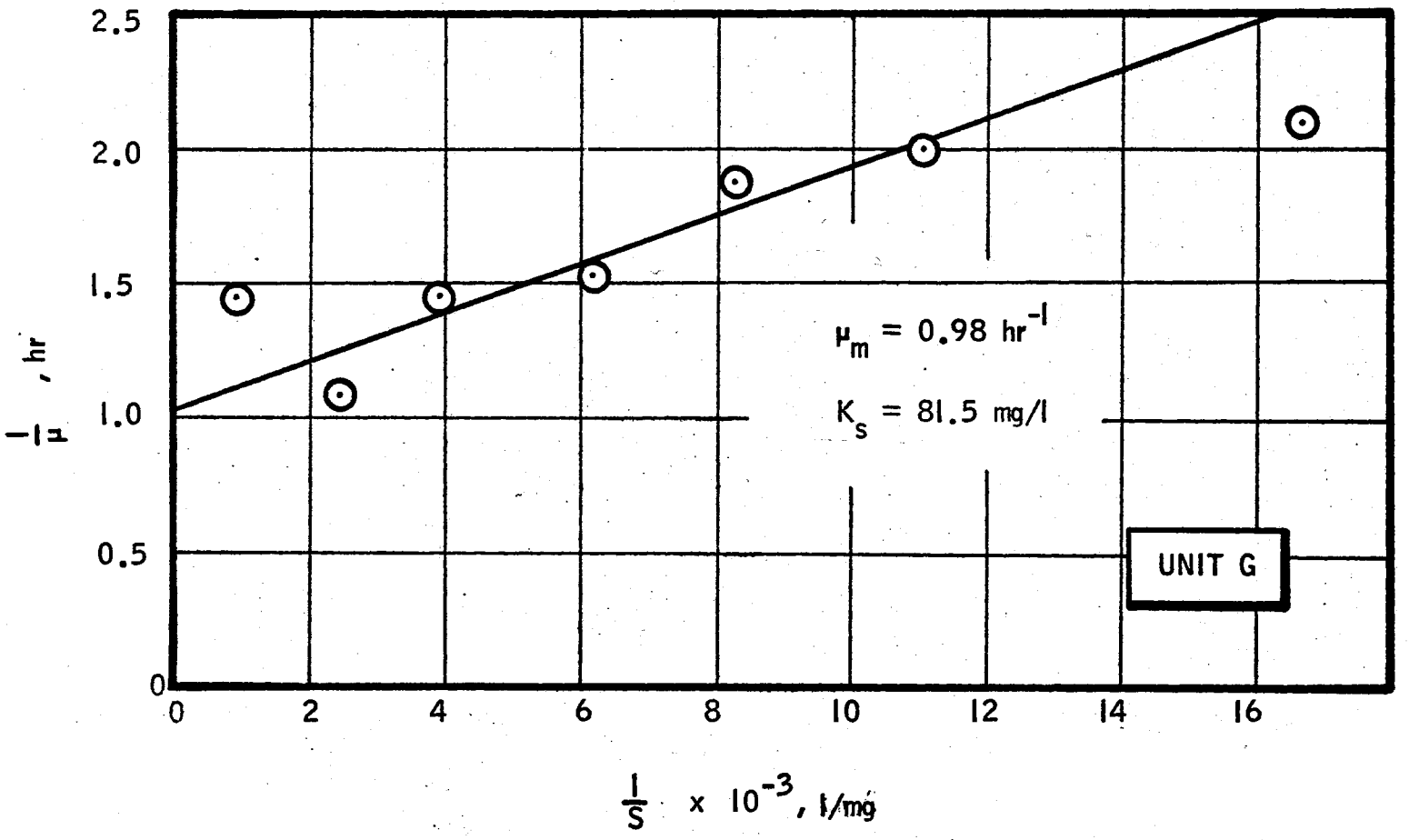

Figure 42. Lineweaver, Burk plot for Experiment No. 7.

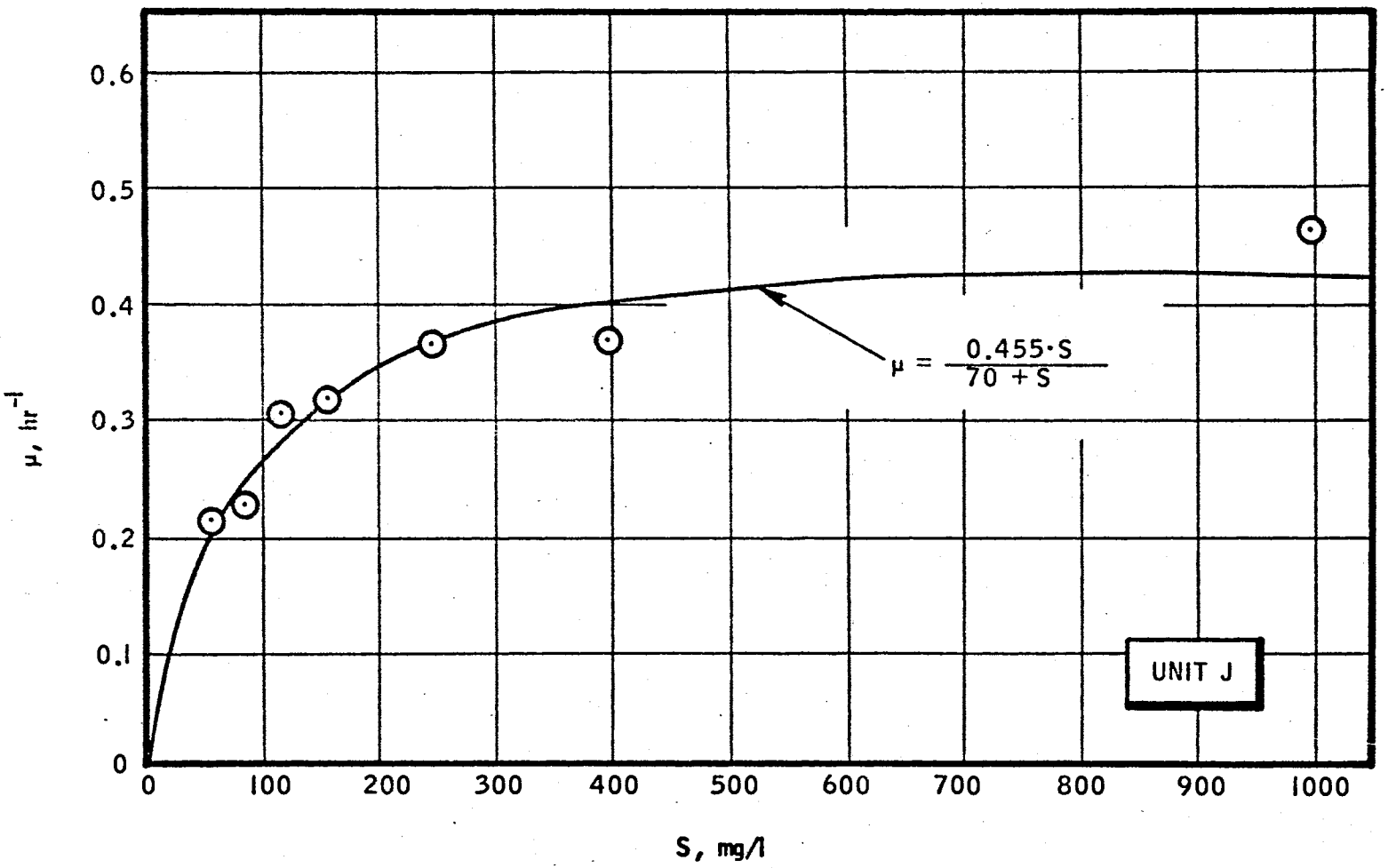

Figure 43. "Monod" plot for Experiment No. 8.

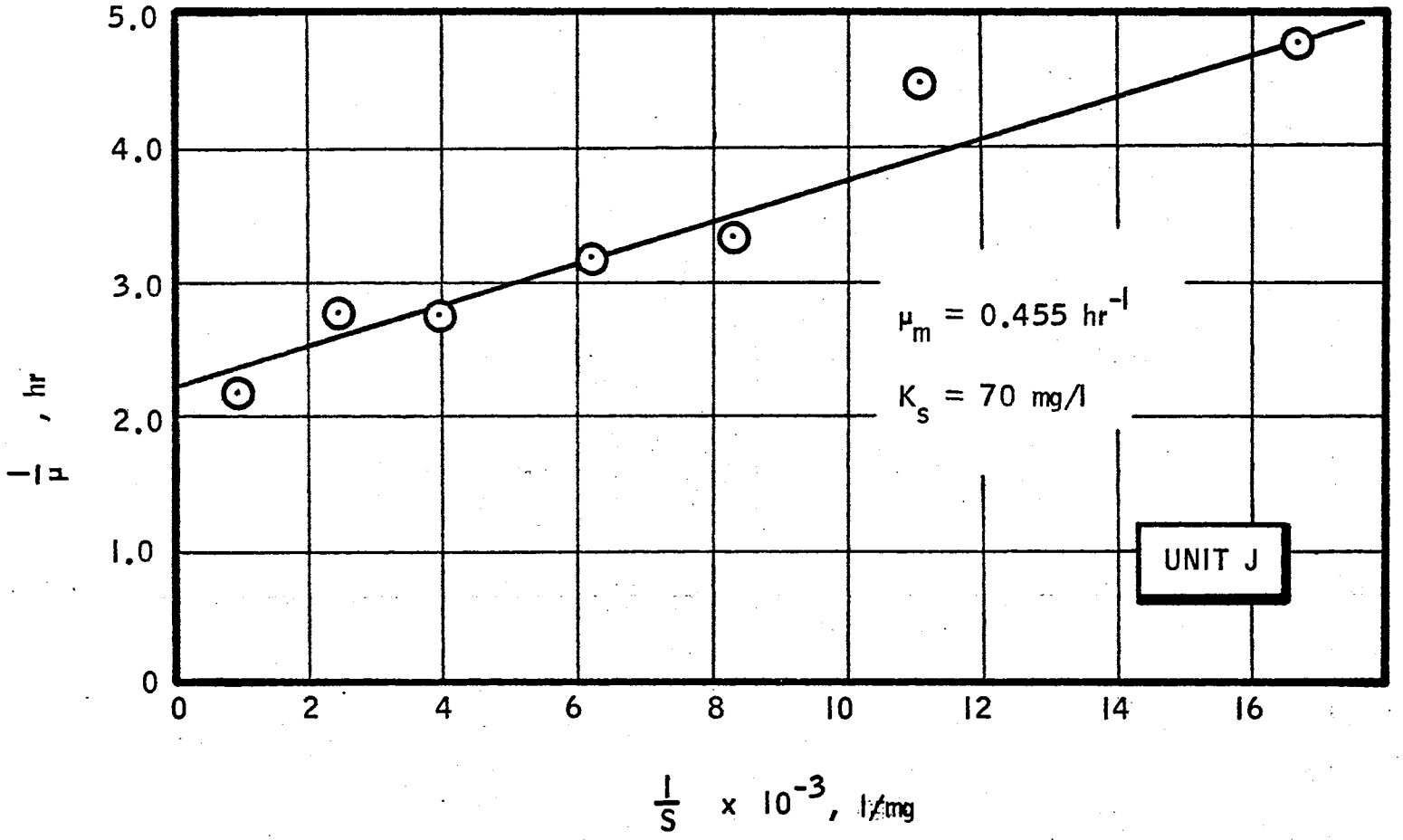

Figure 44. Lineweaver, Burk plot for Experiment No. 8.

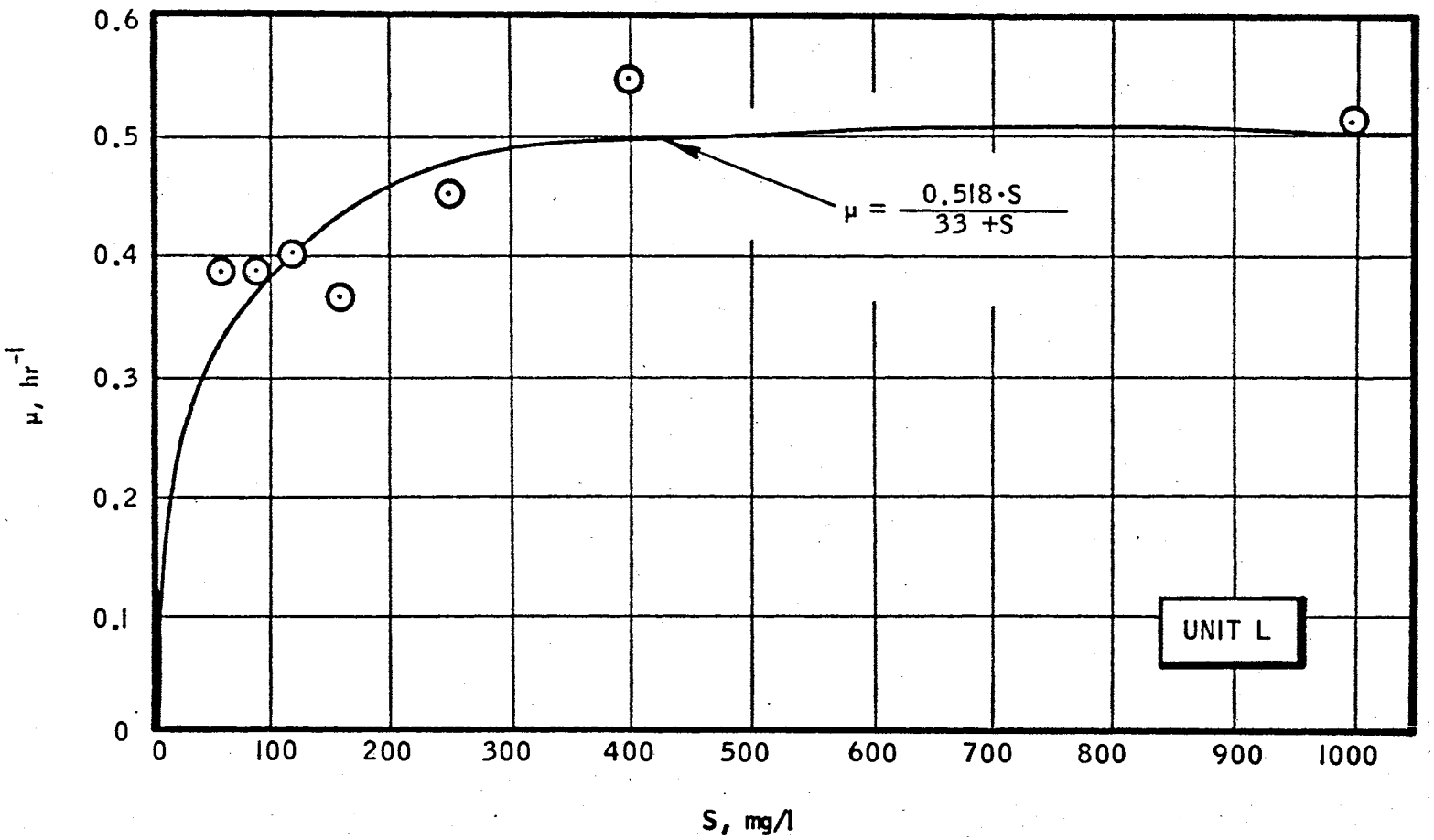

Figure 45. "Monod" plot for Experiment No. 10.

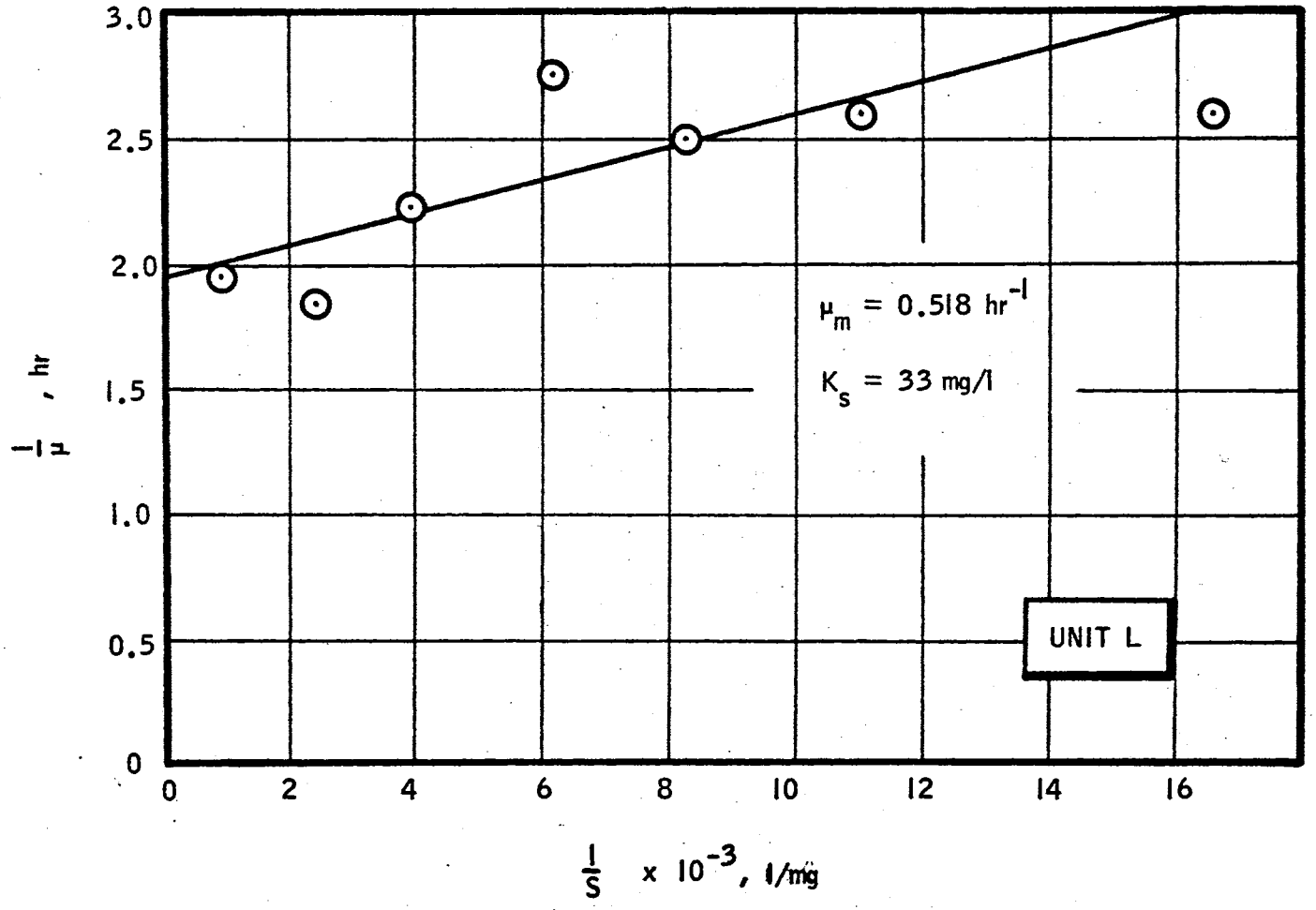

Figure 46. Lineweaver, Burk plot for Experiment No. 10.

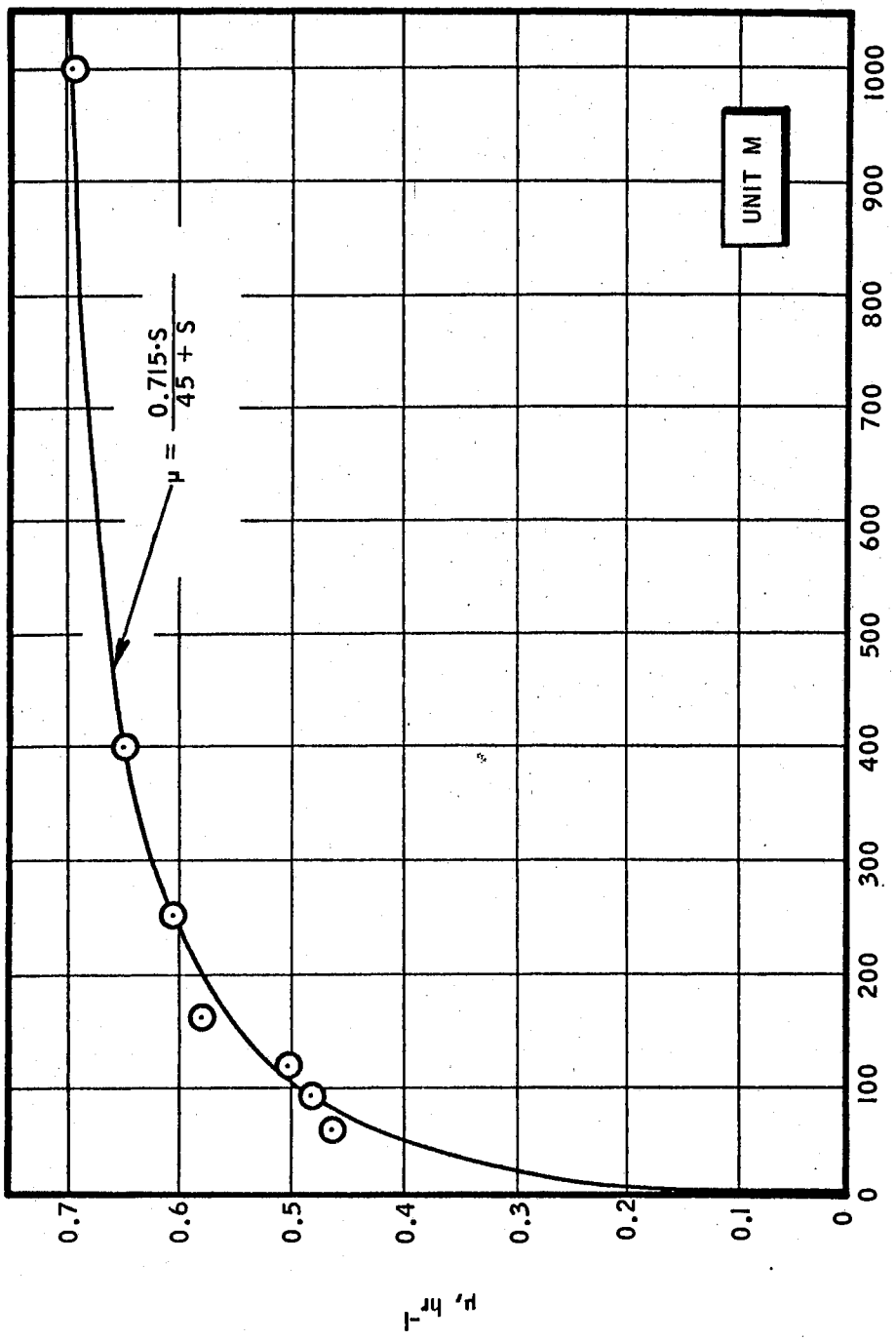

Figure 47. "Monod" plot for Experiment No. 11.

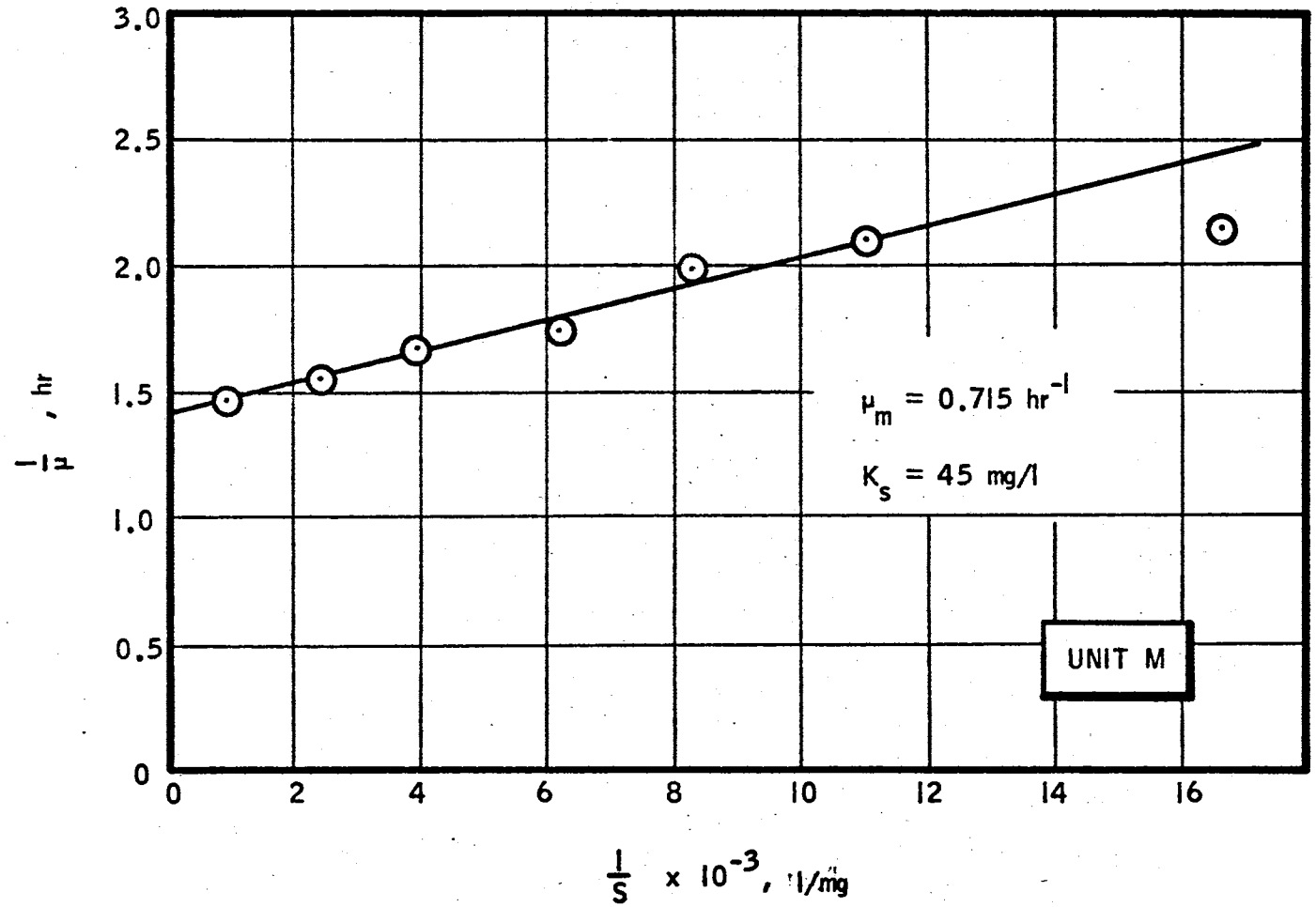

Figure 48. Lineweaver, Burk plot for Experiment No. 11.

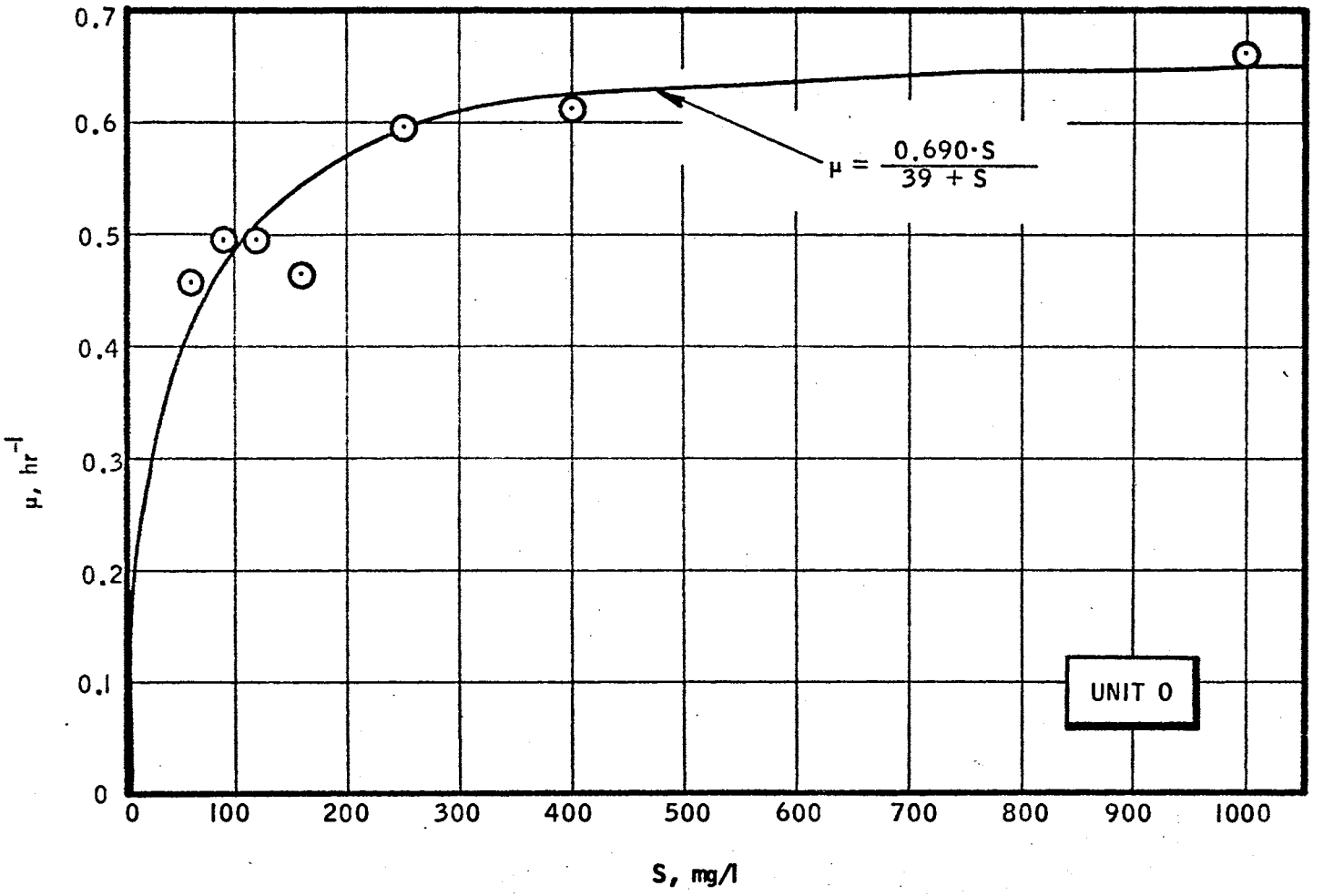

Figure 49. "Monod" plot for Experiment No. 13.

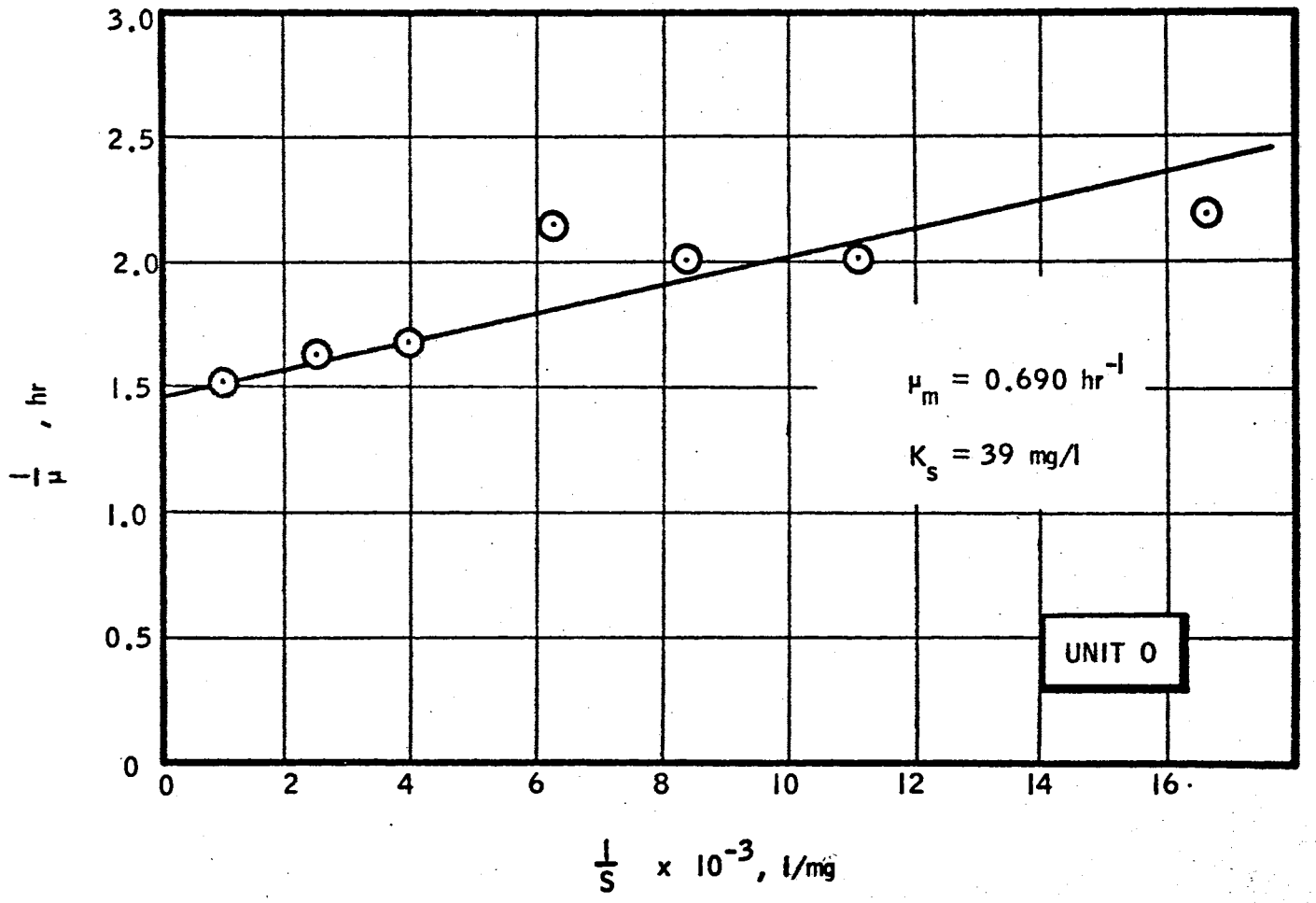

Figure 50. Lineweaver, Burk plot for Experiment No. 13.

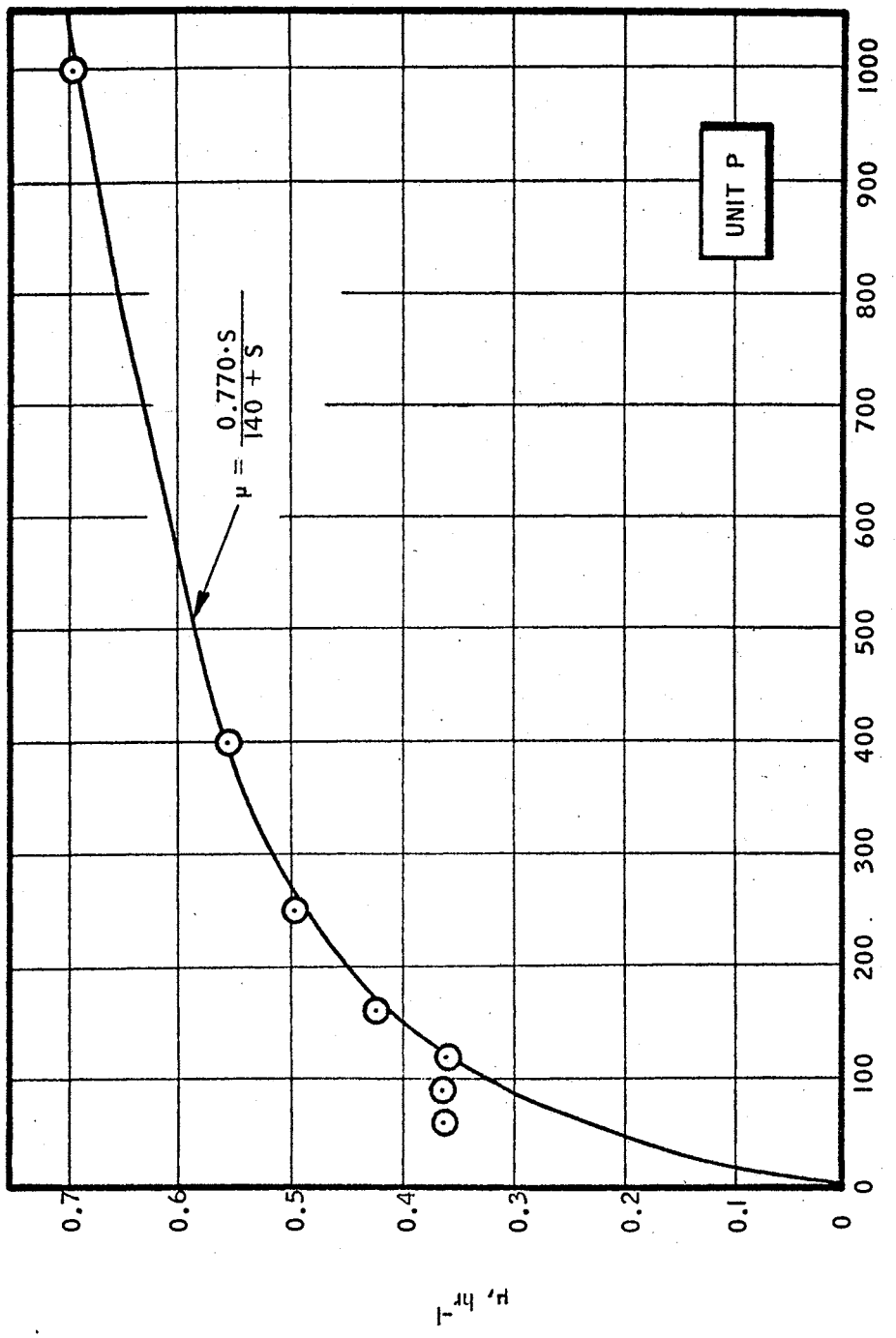

Figure 51. "Monod" plot for Experiment No. 14.

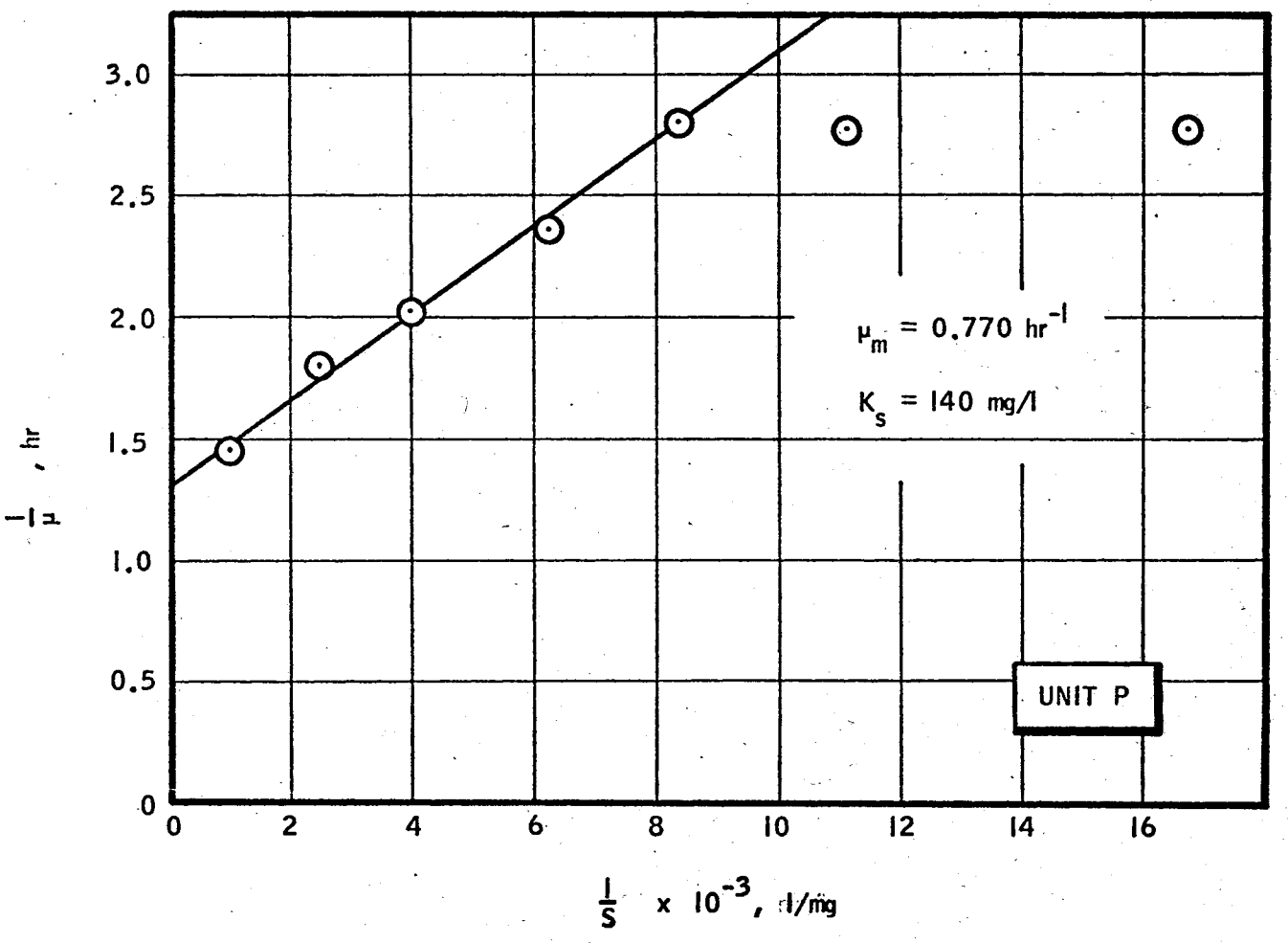

Figure 52. Lineweaver, Burk plot for Experiment No. 14.

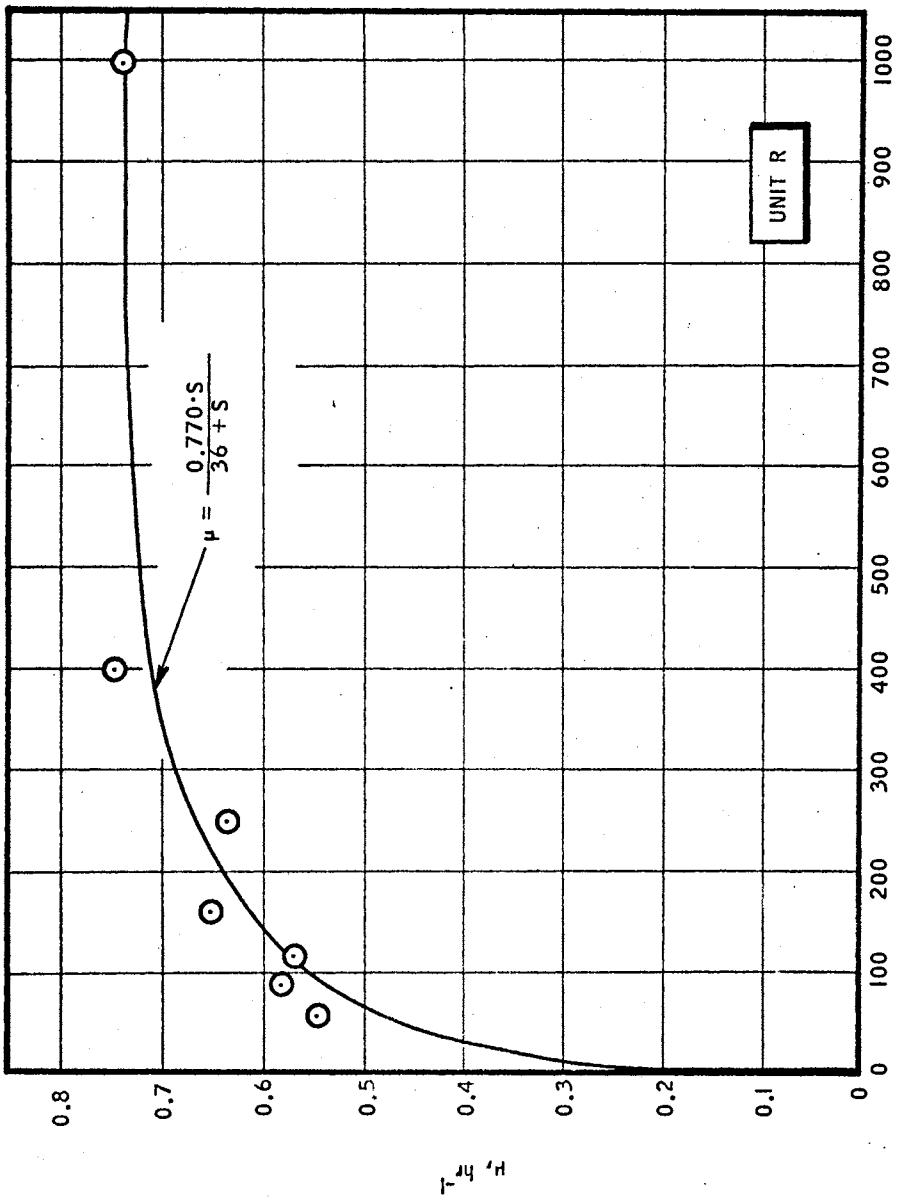

Figure 53. "Monod" plot for Experiment No. 16.

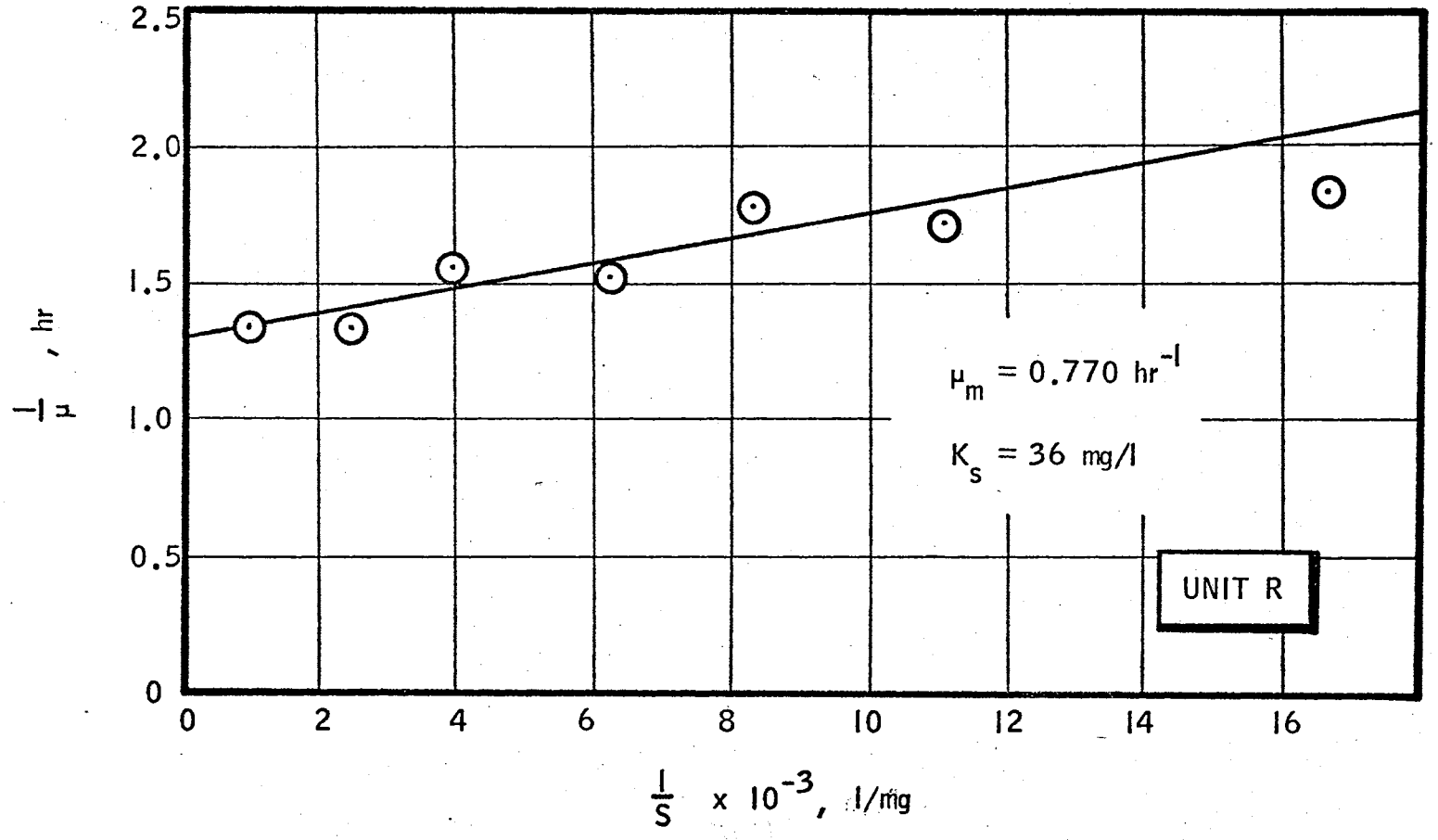

Figure 54. Lineweaver, Burk plot for Experiment No. 16.

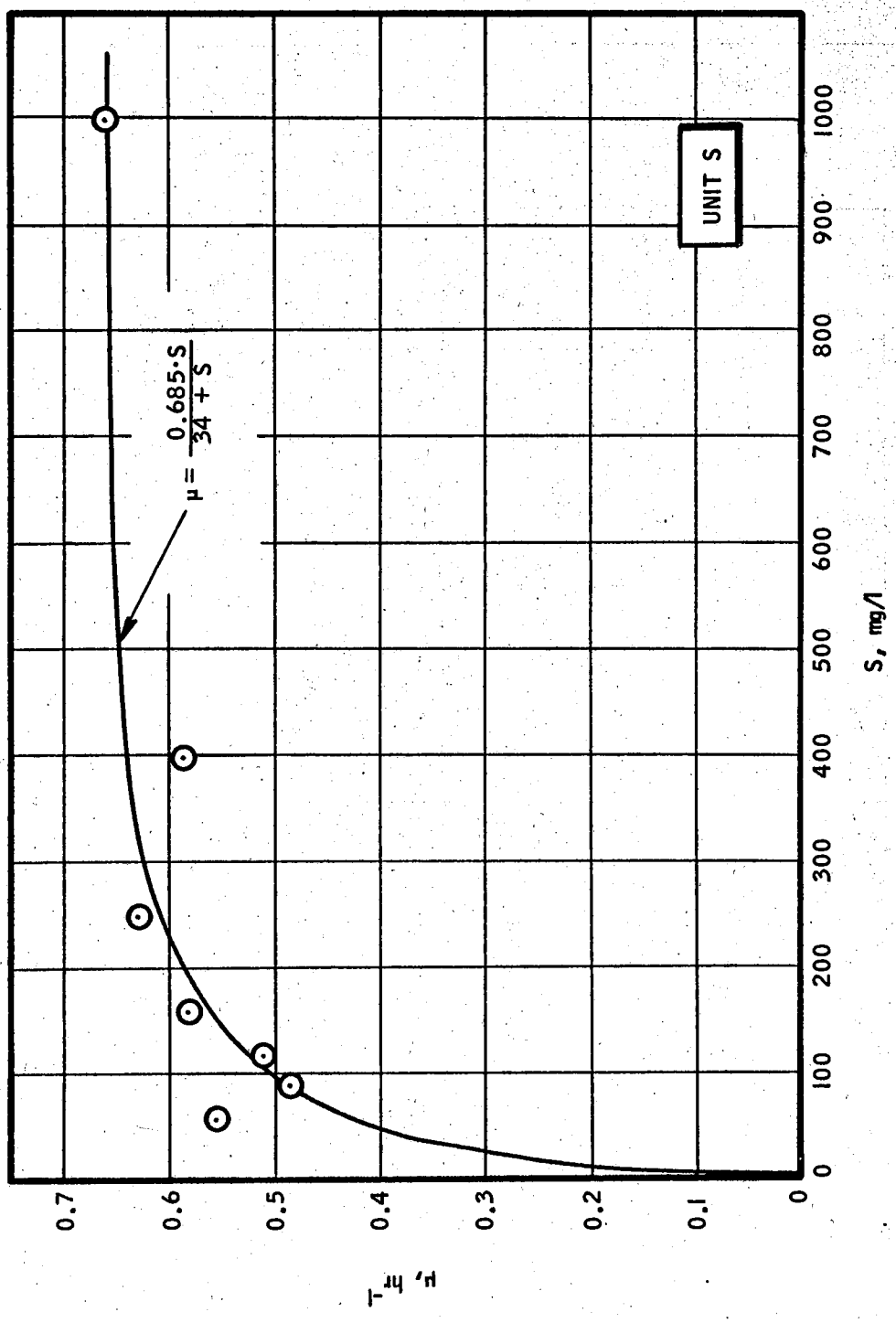

Figure 55. "Monod" plot for Experiment No. 17.

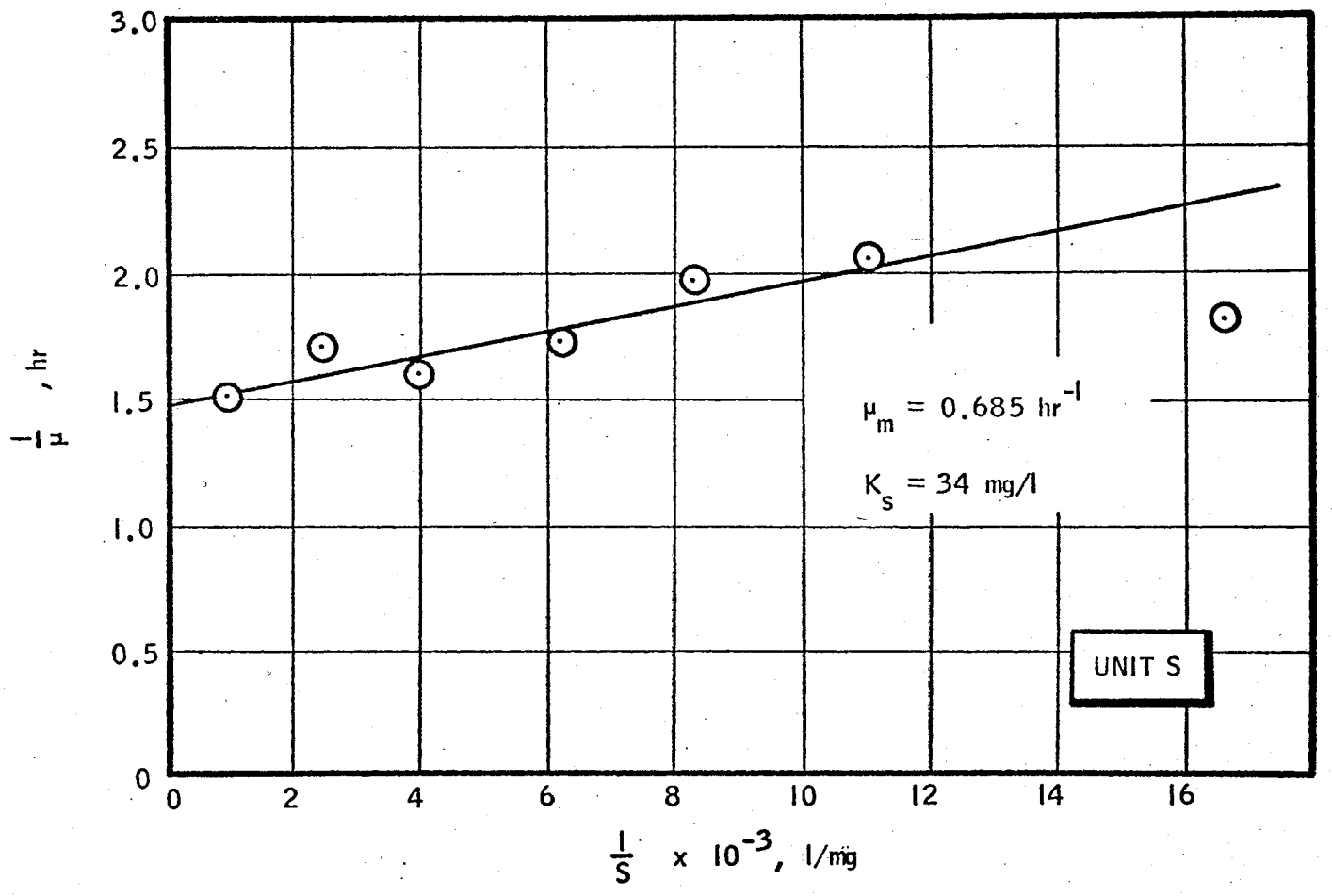

Figure 56. Lineweaver, Burk plot for Experiment No. 17.

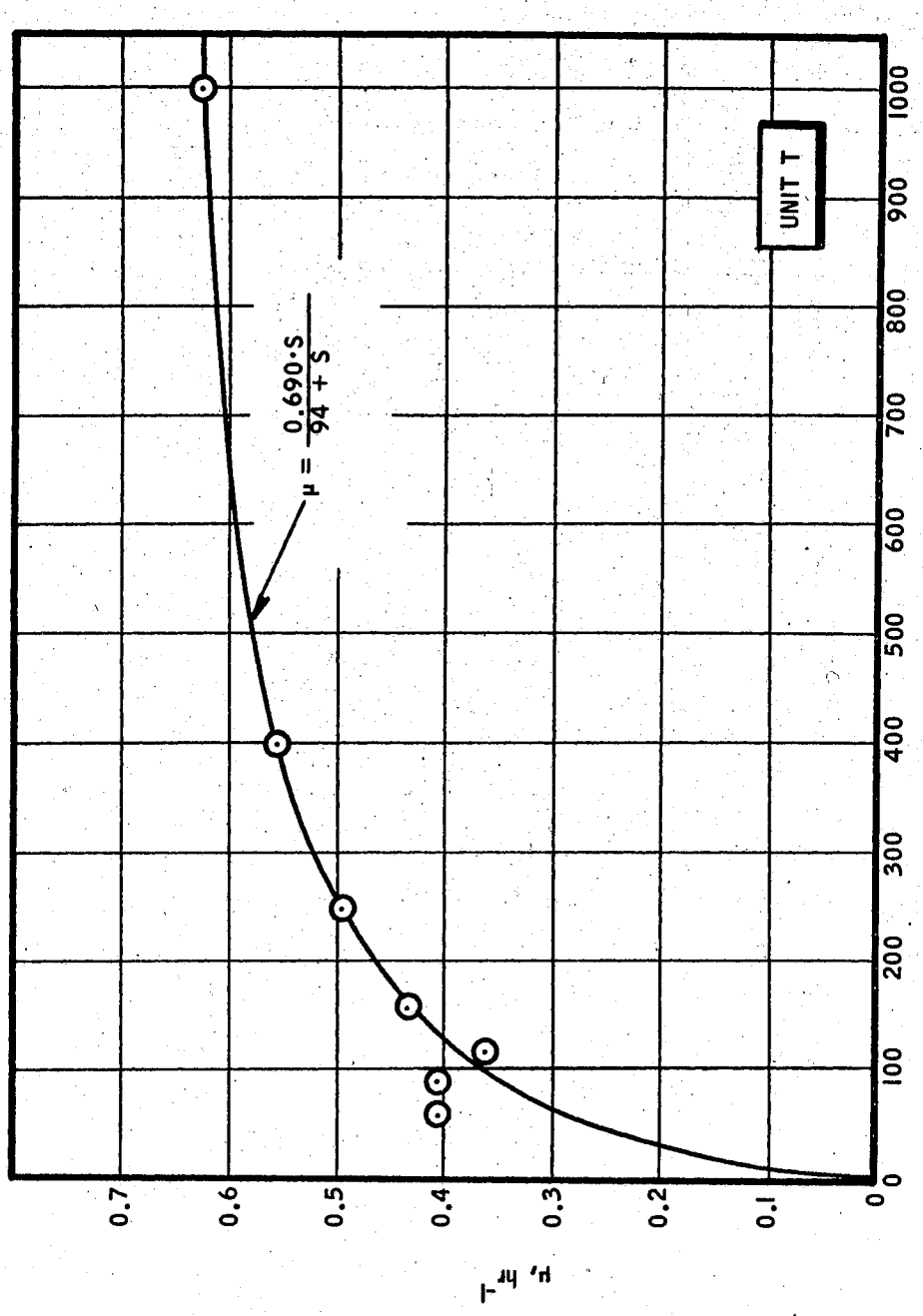

Figure 57. "Monod" plot for Experiment No. 18.

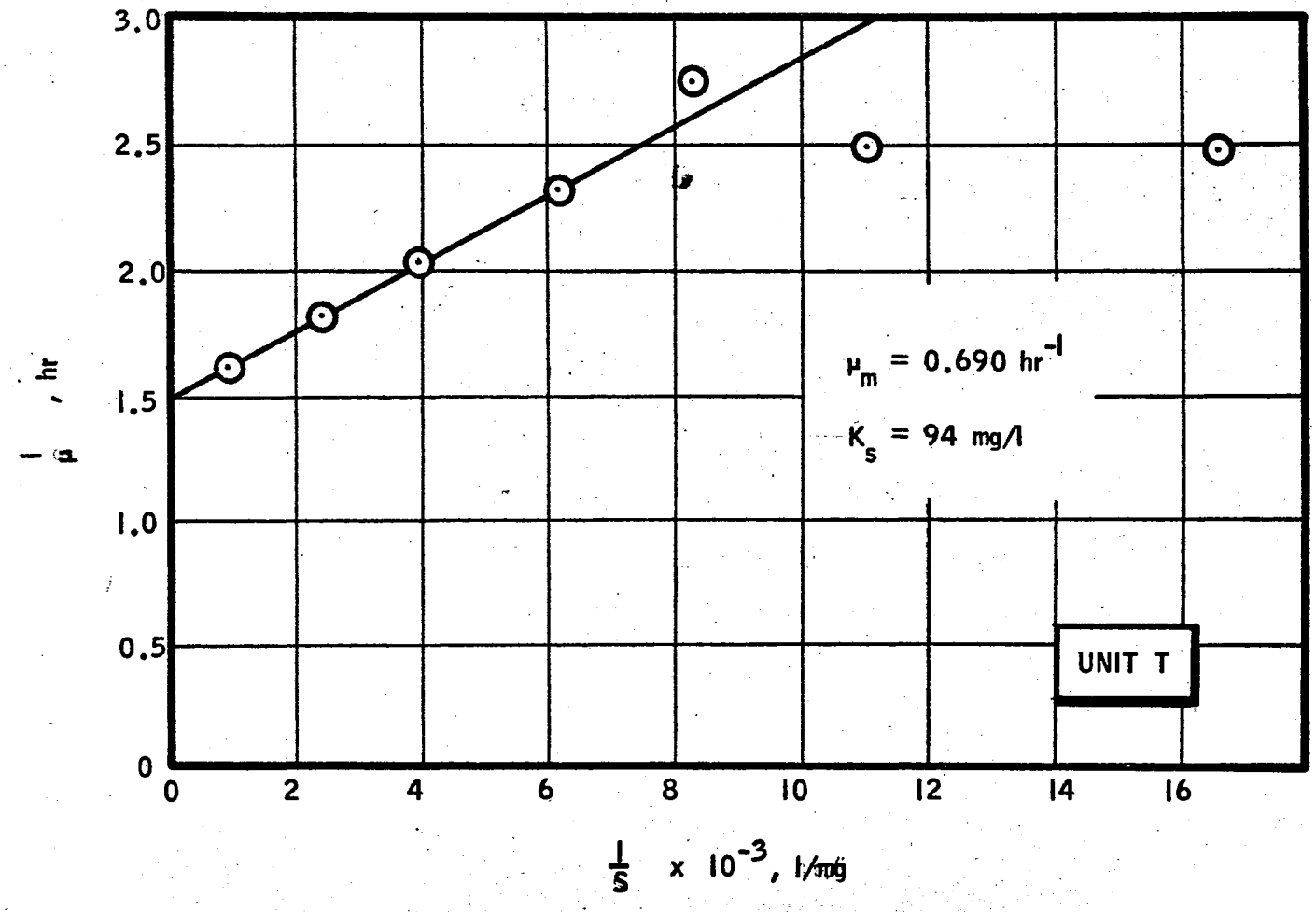

Figure 58. Lineweaver, Burk plot for Experiment No. 18.

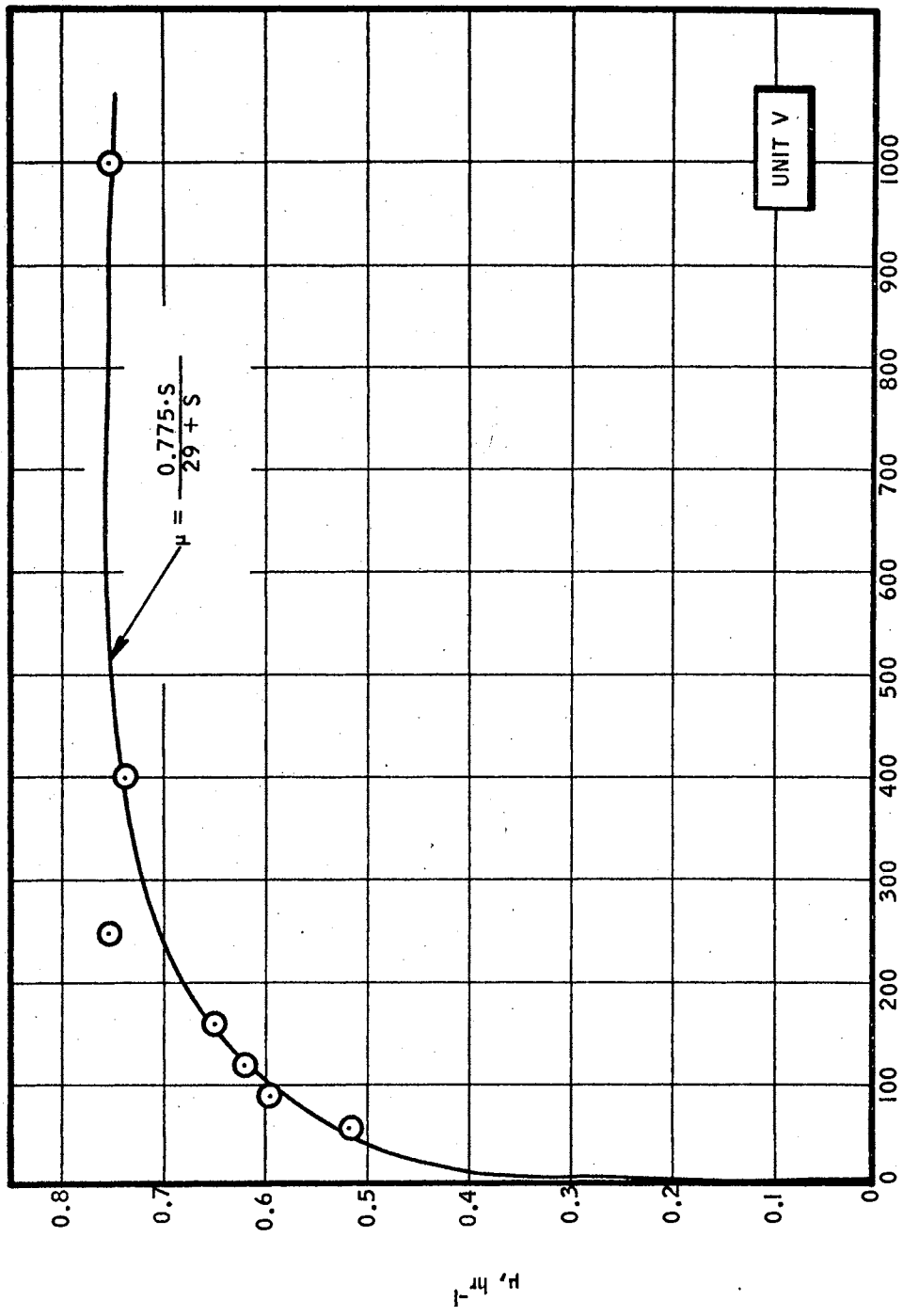

Figure 59. "Monod" plot for Experiment No. 19.

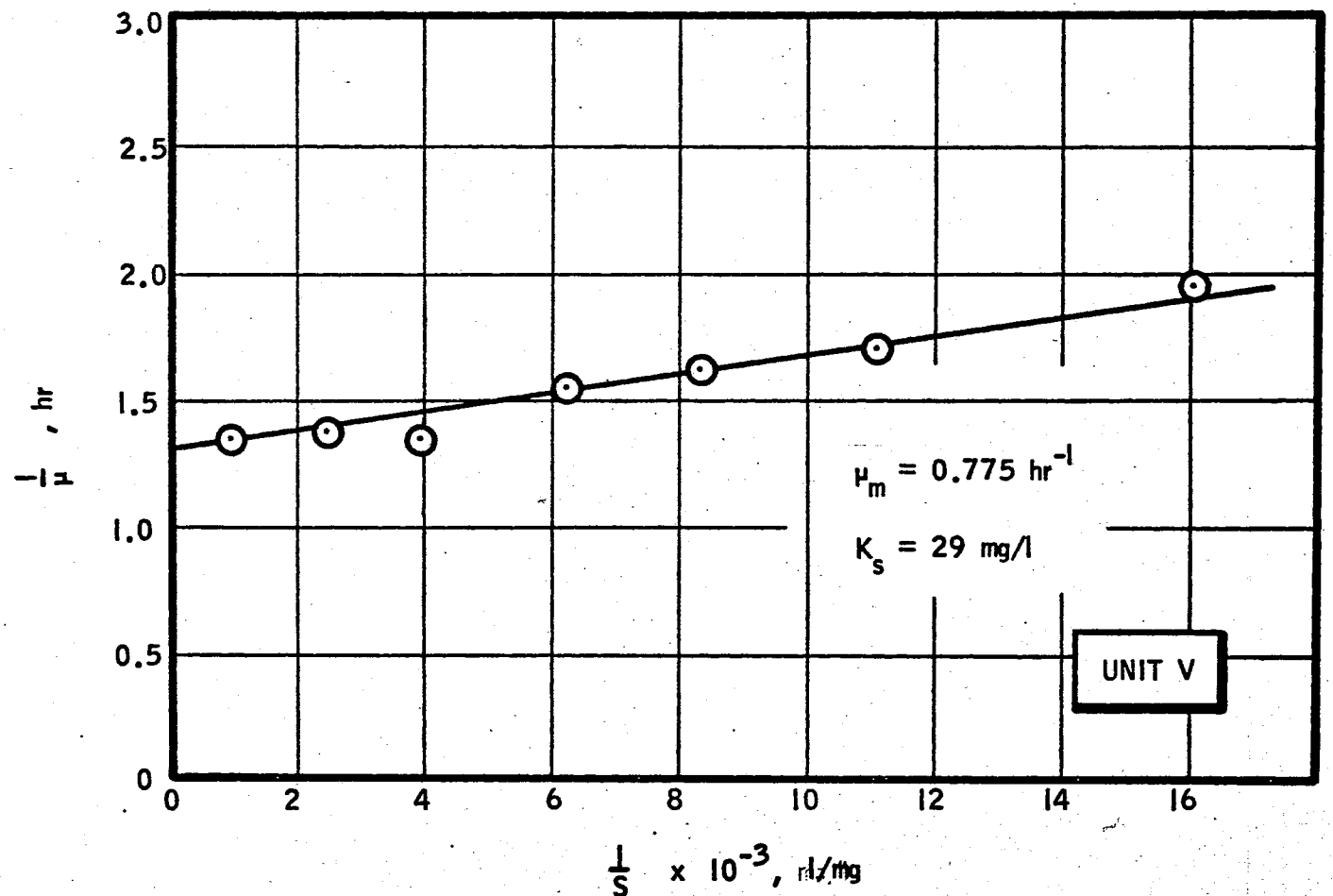

Figure 60. Lineweaver, Burk plot for Experiment No. 19.

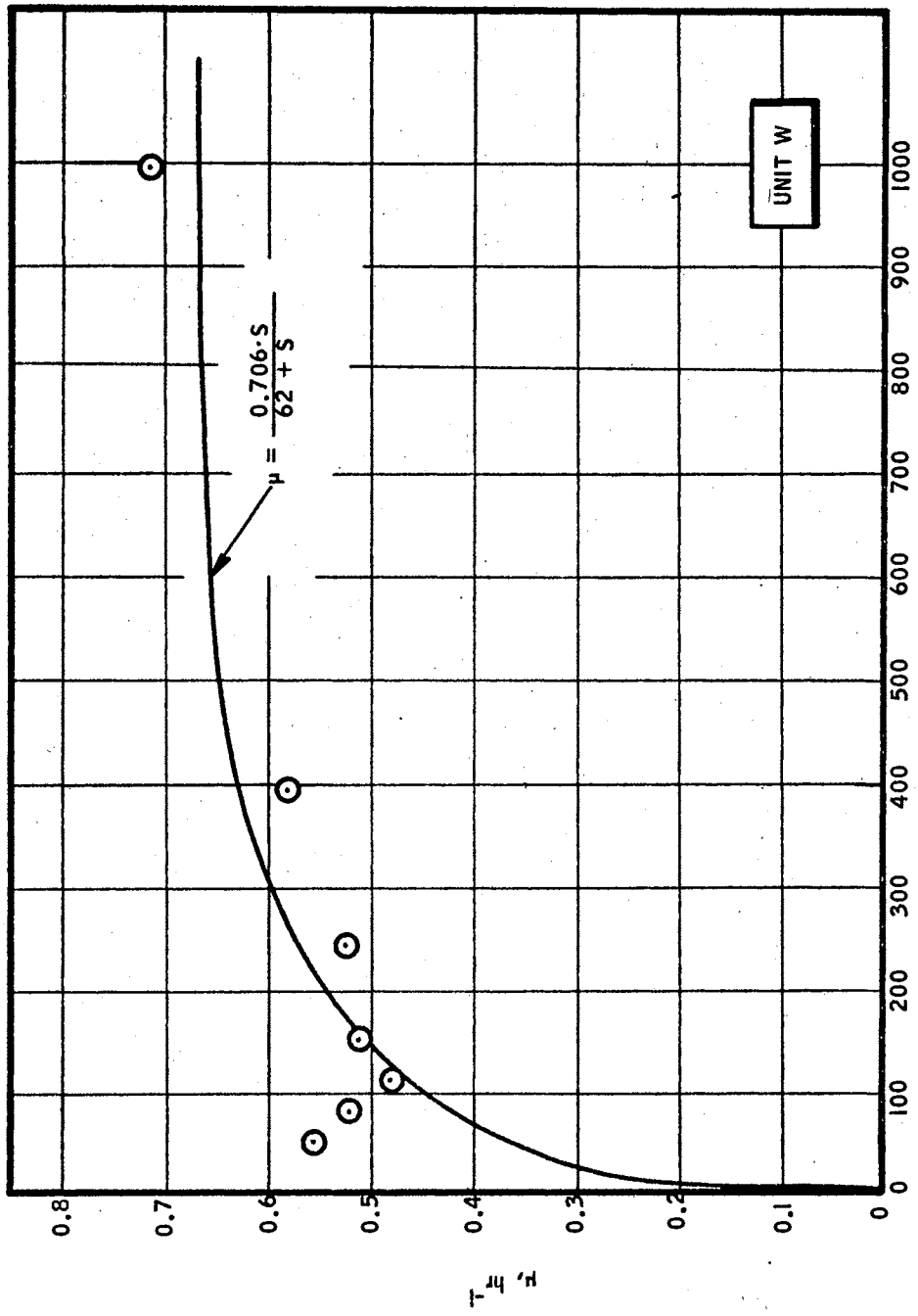

Figure 61. "Monod" plot for Experiment No. 20.

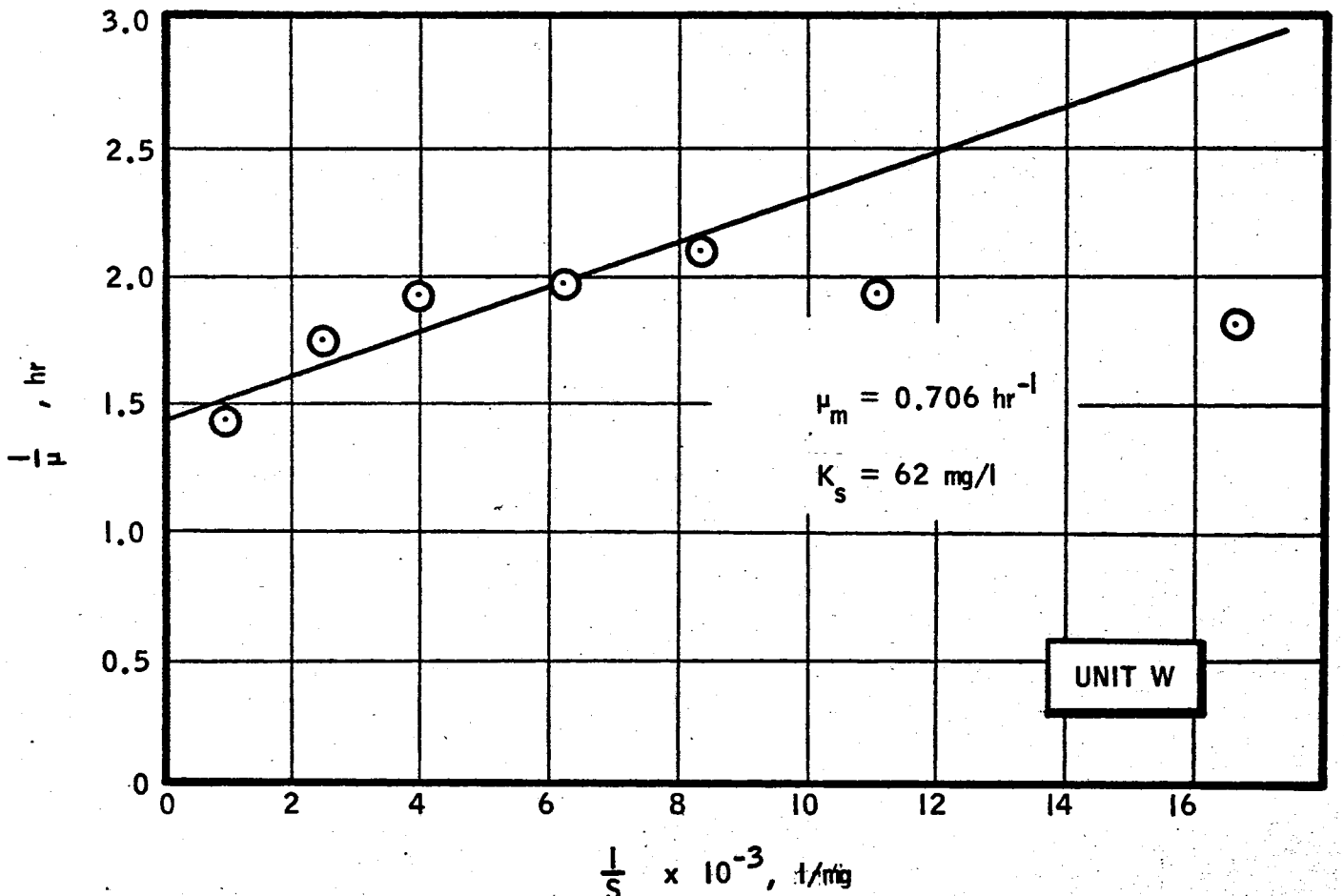

Figure 62. Lineweaver, Burk plot for Experiment No. 20.

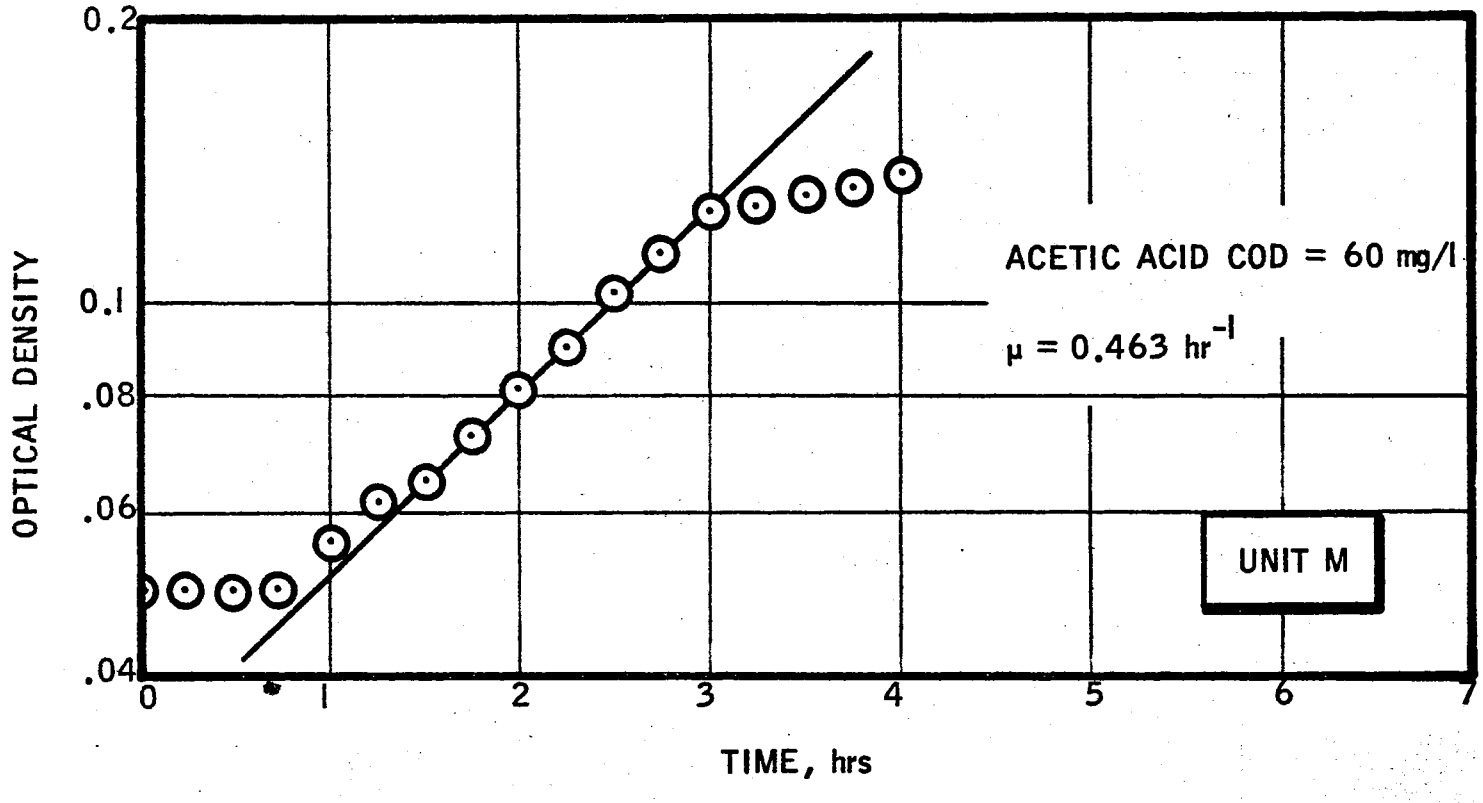

Figure 63. Growth curve for heterogeneous microbial population growing on acetic acid at an initial concentration of 60 mg/l.

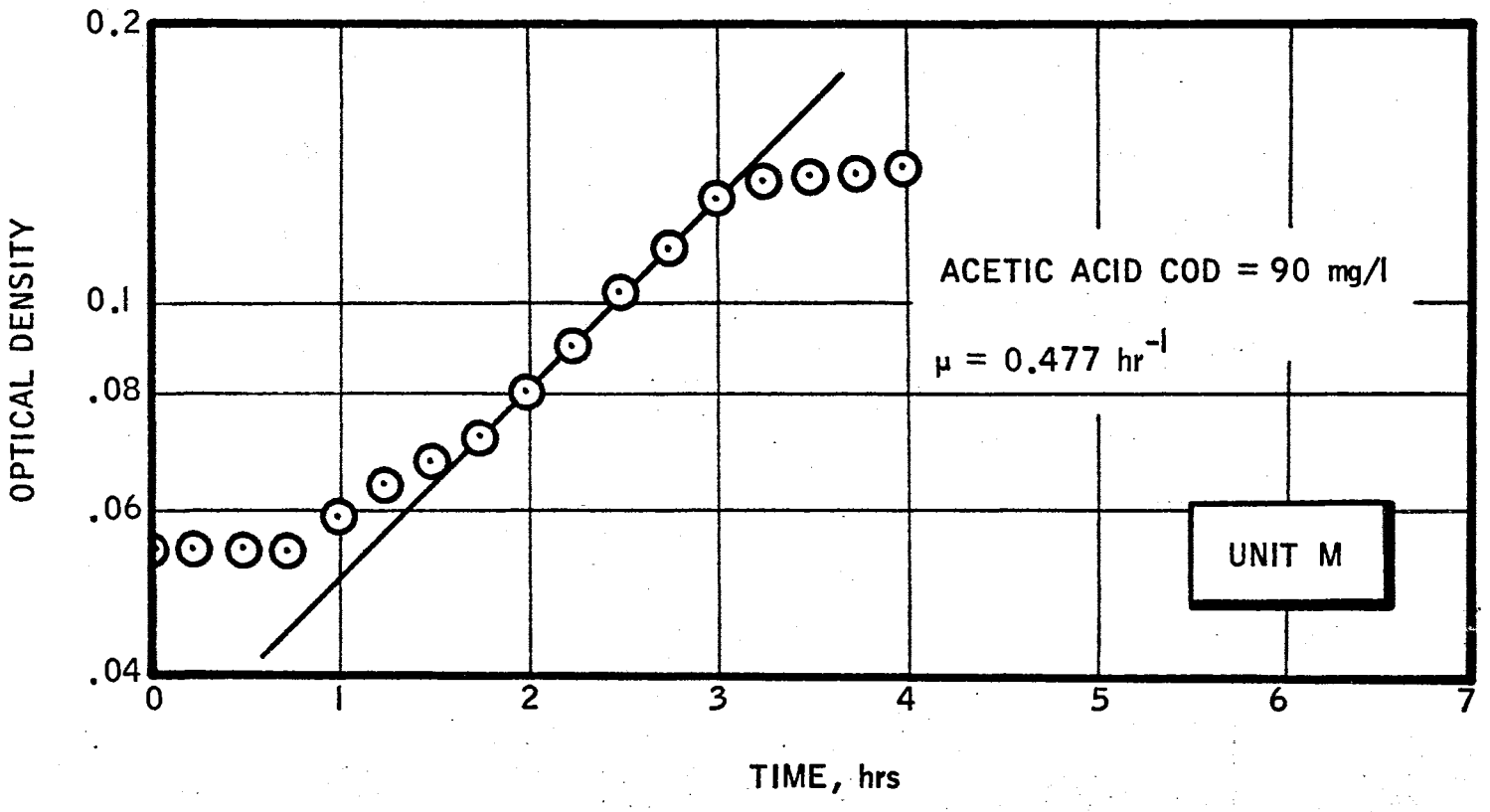

Figure 64. Growth curve for heterogeneous microbial population growing on acetic acid at an initial concentration of 90 mg/l.

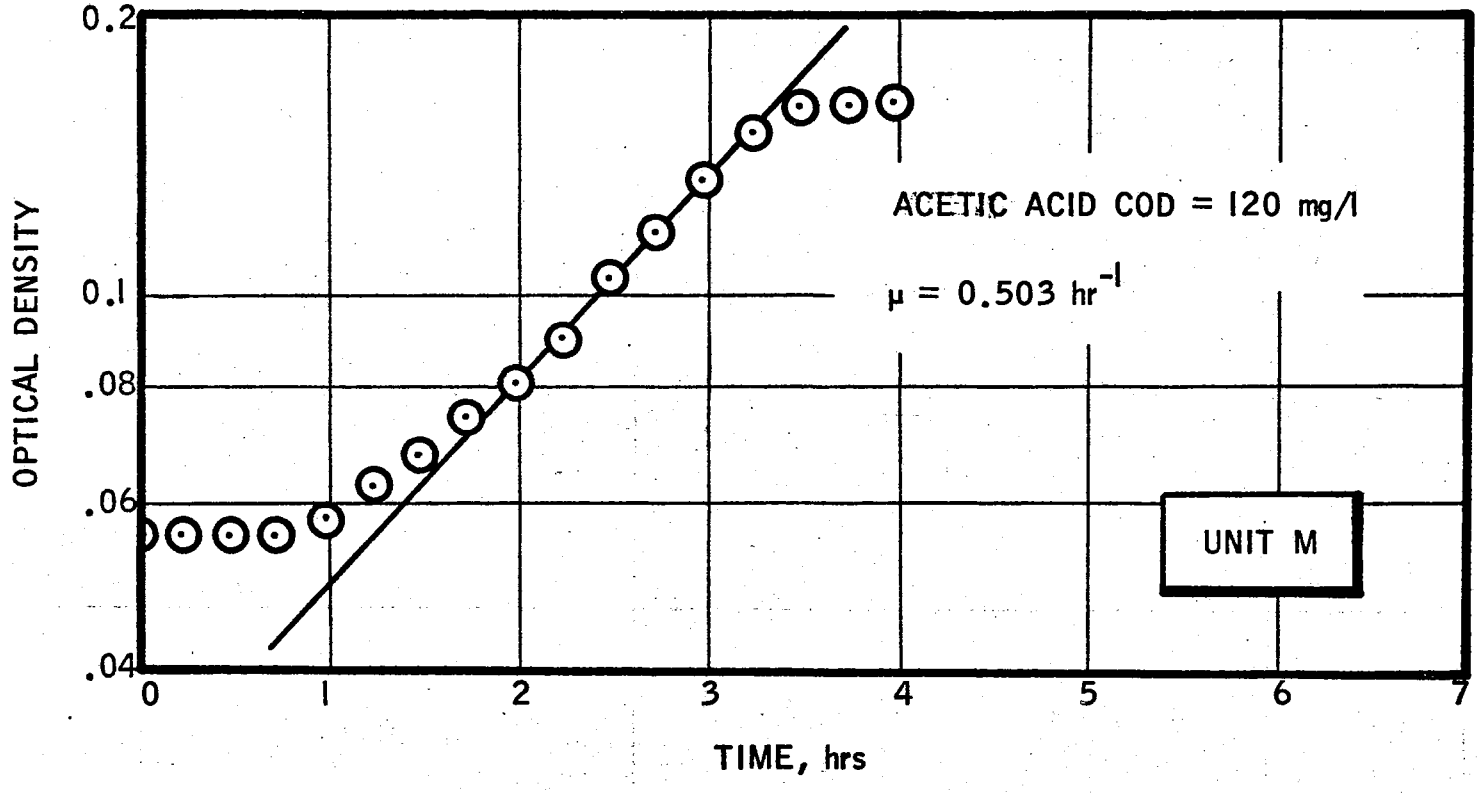

Figure 65. Growth curve for heterogeneous microbial population growing on acetic acid at an initial concentration of 120 mg/l.

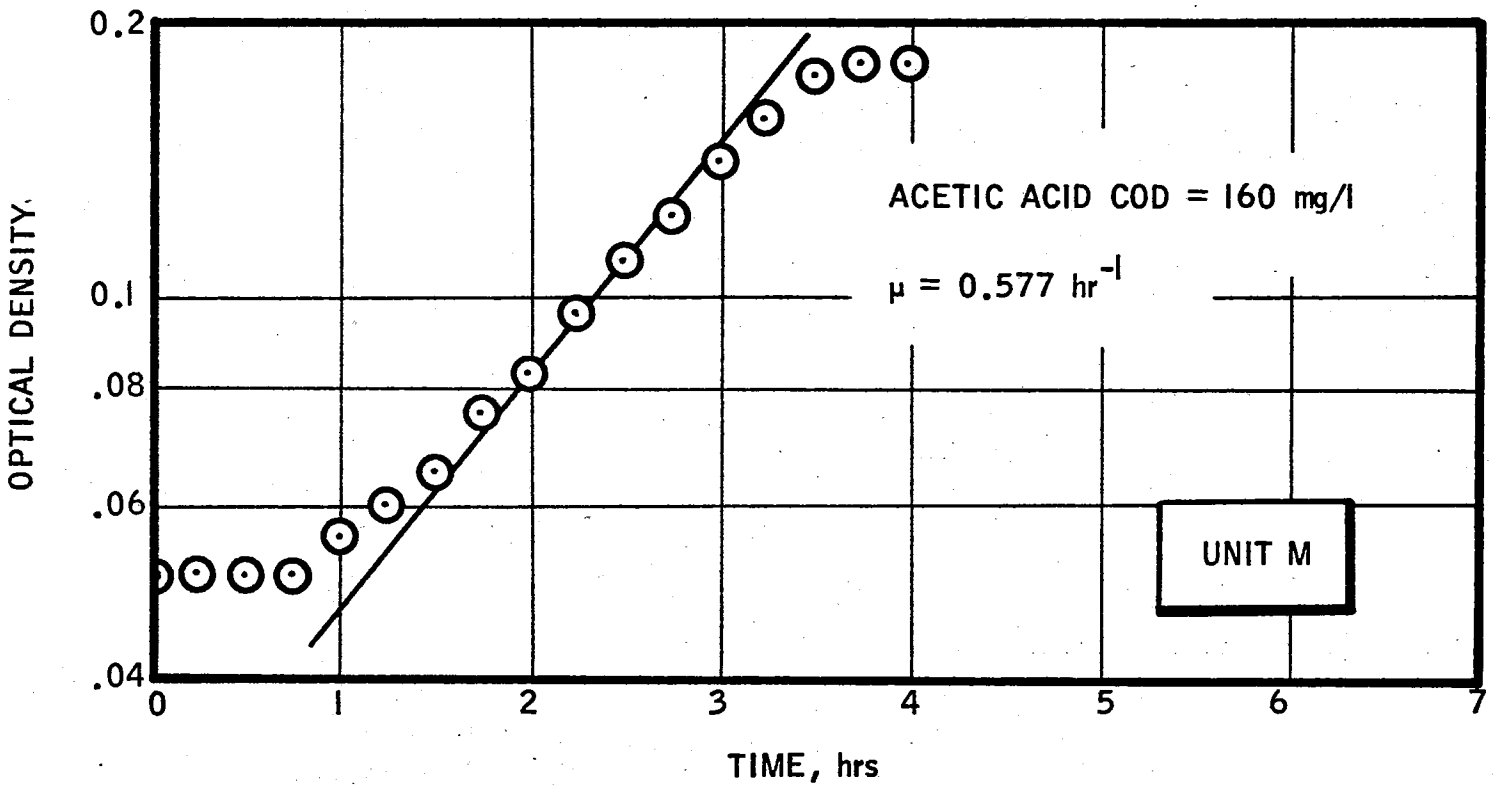

Figure 66. Growth curve for heterogeneous microbial population growing on acetic acid at an initial concentration of 160 mg/l.

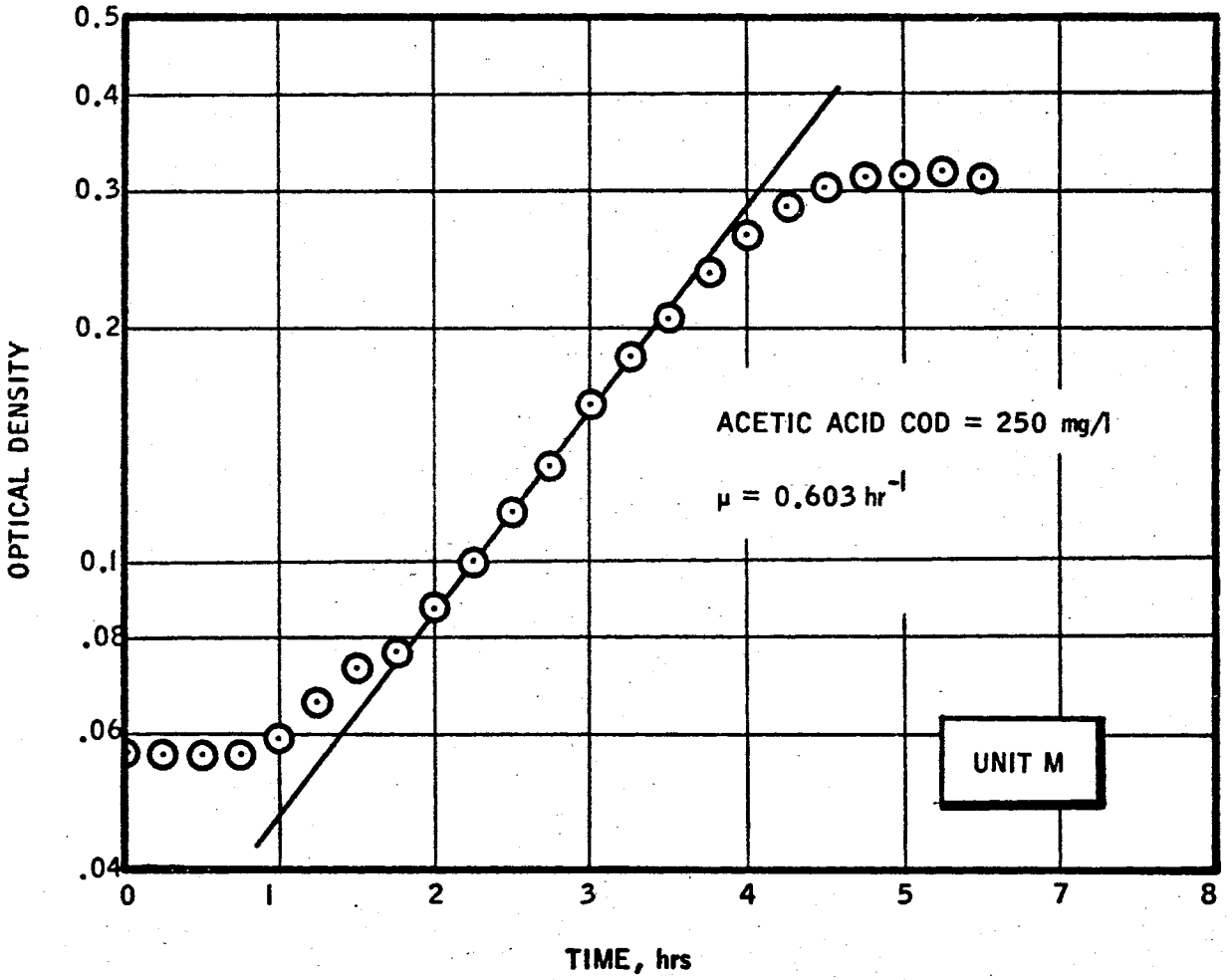

Figure 67. Growth curve for heterogeneous microbial population growing on acetic acid at an initial concentration of 250 mg/l.

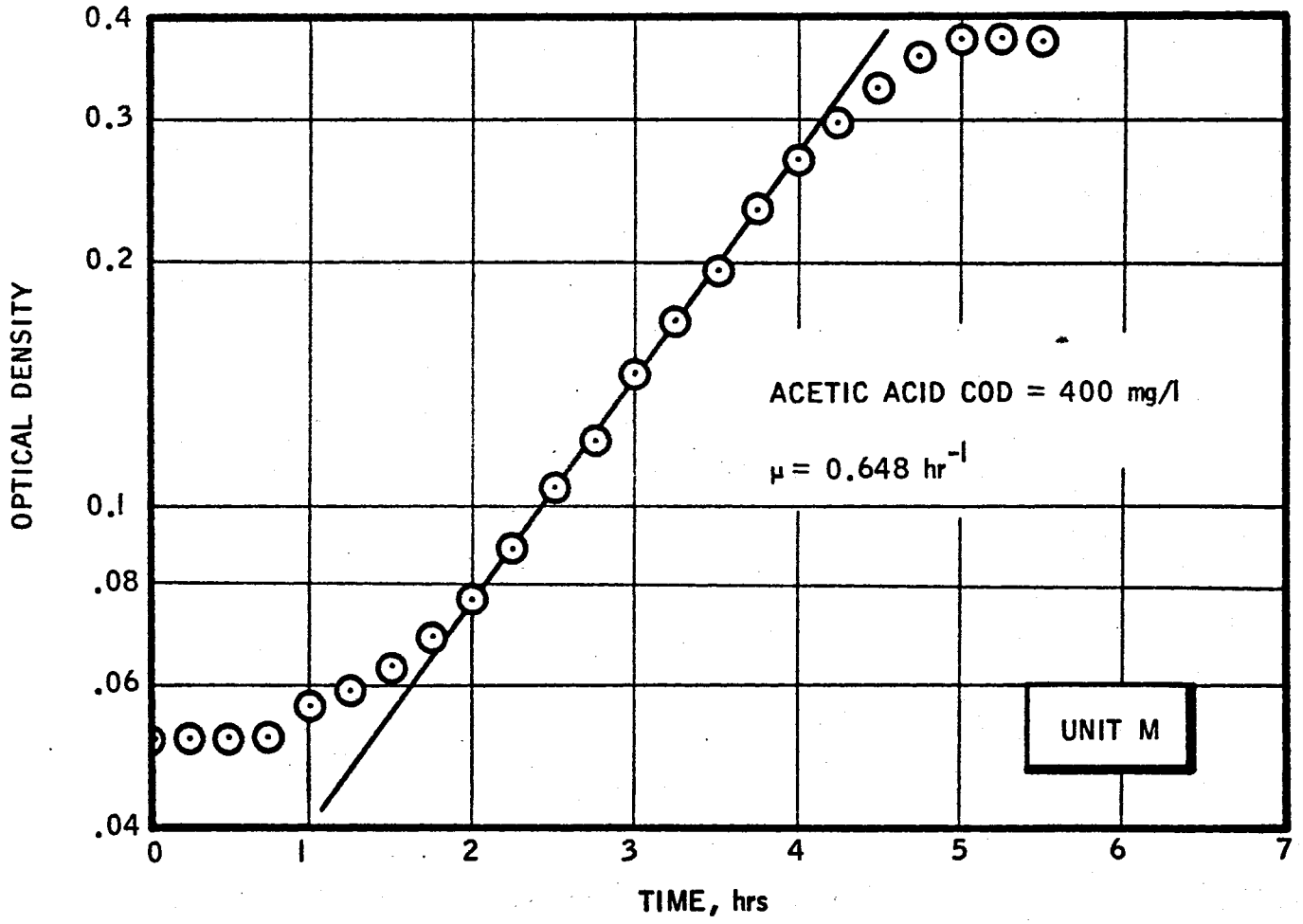

Figure 68. Growth curve for heterogeneous microbial population growing on acetic acid at an initial concentration of 400 mg/l.

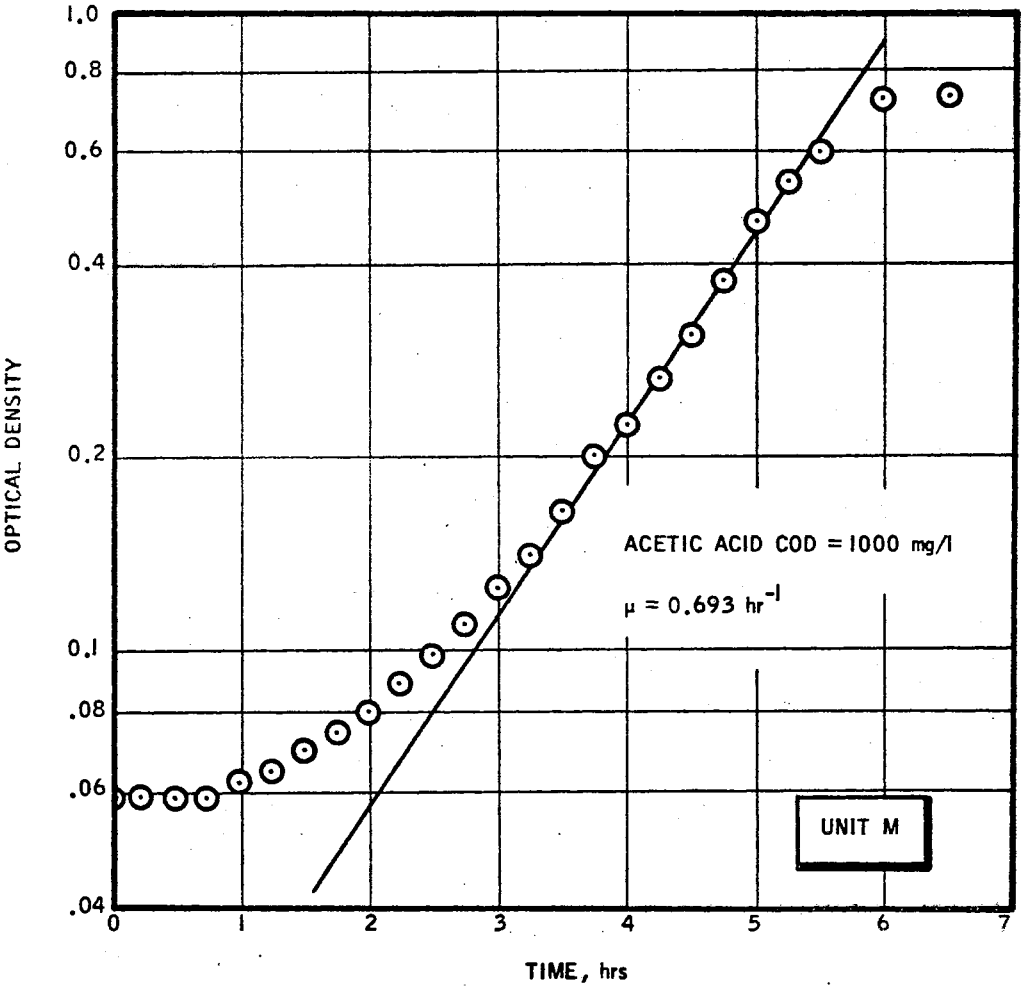

Figure 69. Growth curve for heterogeneous microbial population growing on acetic acid at an initial concentration of 1000 mg/l.

the slope of a straight-line plot of optical density [log] vs. time) can occur at substrate concentrations below maximum growth rate. A statistical analysis of maximum growth rate and saturation constant values is shown in Table IV. The values of growth parameters for their respective experiments are found in Tables II and III.

C. Endogenous Metabolism

Figures 1-20 show the metabolic activity of activated sludge mixed populations during prolonged endogenous aeration conditions. The "total oxidation" of solids accumulated during the substrate removal phase was observed in many of the batch activated sludge-extended aeration systems (see section I). It can be seen in experiments 8, 9, 10, 13, 14, and 16 (Figures 8, 9, 10, 13, 14, and 16) that all of the protein synthesized during the substrate removal phase was oxidized during endogenous metabolism. Figures 12, 15, 18, and 19 show almost "total oxidation" of endogenous protein during the aerobic auto-oxidative phase. Results of experiments 8-20 (Figures 8-20) show carbohydrate not to be a primary endogenous reserve material, and consequently not a main endogenous carbon source. Figures 1-20 show the residual soluble organic matter to remain relatively constant, and in most experiments, concentrations varying between two and ten percent of the theoretical COD are observed. Tables V and VI present a summary of sludge characteristics in the endogenous phase for all acetic acid systems.

TABLE V

SUMMARY OF SLUDGE CHARACTERISTICS IN THE ENDOGENOUS PHASE OF ACETIC ACID SYSTEMS 1-7

Exper. No.	Fig. No.	Time (Hrs.)	Sludge				
				Final Synthesis			
			Final Conc. mg/l	Weight mg/l	Yield Percent	As % Sludge Synthesized	As % of Theoretical COD
1	1	312	110	80	8.2	20.5	7.5
2	2	312	74	26	2.6	7.1	2.5
3	3	312	78	0	0	0	0
4	4	312	53	0	0	0	0
5	5	312	93	38	4.1	8.9	3.6
6	6	312	58	3	0.32	0.68	0.28
7	7	312	125	85	9.4	22.4	8.0

TABLE VI

SUMMARY OF SLUDGE CHARACTERISTICS IN THE ENDOGENOUS PHASE OF ACETIC ACID SYSTEMS 8-20

Exper. No.	Fig. No.	Time* (hrs.)	Sludge					Sludge Carbohydrate		Sludge Protein	
			Final Conc. (mg/l)	Final Synthesis				Final Content		Final Content	
				Weight (mg/l)	Yield Percent	As % Sludge Synthesized	As % of Theoretical COD	Time† (hrs.)	Weight (mg/l)	As % Sludge Dry Wt.	Weight (mg/l)	As % Sludge Dry Wt.
8	8	328	50	5	0.5	1.5	0.4	280	10	20.0	9	18.0
9	9	328	58	0	0	0	0	280	13	22.4	29	50.0
10	10	312	60	0	0	0	0	264	9	15.0	24	40.0
11	11	360	100	75	7.8	20.7	7.0	336	18	18.0	50	50.0
12	12	360	92	67	6.8	20.1	6.3	336	14	15.2	37	40.2
13	13	324	80	40	4.0	12.5	3.7	324	8	10.0	18	22.5
14	14	340	65	17	1.7	5.0	1.6	340	7	10.7	20	30.7
15	15	312	90	40	4.2	11.8	3.7	312	5	5.5	30	33.3
16	16	336	150	87	8.7	25.5	8.2	336	9	6.0	20	13.3
17	17	600	185	165	17.7	44.2	15.5	600	20	10.8	50	27.0
18	18	600	100	55	5.5	17.1	5.1	552	10	10.0	25	25.0
19	19	600	90	75	7.0	22.8	7.0	408	9	10.0	25	27.7
20	20	840	160	102	10.7	28.7	9.6	316	24	15.0	71	44.3

*Time at which the experiment was terminated.

†Last sampling time for determination of cell protein and cell carbohydrate.

CHAPTER VI

DISCUSSION

A. Metabolism of Acetic Acid for All Activated Sludge-Extended Aeration Systems

The results for twenty long-term once-fed batch experiments on activated sludge consisting of acclimated natural microbial populations growing on acetic acid as their sole carbon source and growth-limiting factor were shown in Figures 1-20. A summary of characteristics for all acetic acid systems at time of removal of exogenous substrate was presented in Tables II and III.

It was observed from semi-logarithmic plots of biological solids and optical density obtained during experiments 1-20 that during the substrate removal phase, sludge synthesis underwent a period of exponential increase (first-order increasing rate kinetics). In general, the same was true for substrate removal. It was also seen that, except for experiments 6, 7, and 17 (Figures 6, 7, and 17), times of maximum removal of exogenous substrate and maximum cell growth coincided.

Tables II and III show biochemical efficiencies of the activated sludge systems (experiments 1-18 and 20) to be 93 percent or better. It was also seen from Tables II and III and Figures 21-40 that cell yield, i.e., that portion of organic molecules channelled into synthesis during substrate catabolism varied from 35 to 50 percent.

It is noted that the carbohydrate content of these acetic acid-grown cells was somewhat low. Cognizant of the fact that acetate serves as a biosynthetic precursor for synthesis of fatty acids and other lipid materials, it is suggested here that lipid material, e.g., polyhydroxybuturate, rather than carbohydrate, was a main storage product during the metabolism of acetic acid.

It can be observed from Figures 1-20 and Tables V and VI that under prolonged endogenous aeration, "total oxidation" of an accumulated sludge mass grown on the low molecular weight organic compound acetic acid is definitely possible. During the "endogenous respiration" phase, low concentrations of residual soluble organic matter were observed along with high utilization of proteinaceous material. This phase is discussed extensively in section C.

B. Growth Kinetics

1. Cell Yield

The results seen in Figures 21-40 (experiments 1-20) provide additional research data which confirms the concept that cell yield can be considered constant during the entire course of autocatalytic growth. It was seen (Figures 21-40) that cell yield calculated at the end of the substrate removal phase was essentially the same as cell yield calculated from the slope of a straight line function of biological solids vs. substrate (COD) removed during the substrate removal phase. Thus, sludge yields calculated in the usual manner (end of removal phase) can be validly employed.

The overall results for cell yield analysis (Tables II, III, IV, and Figures 21-40) for the acclimated mixed microbial populations

utilizing a low molecular weight non-carbohydrate compound show a lower portion of the theoretical COD to be used for assimilation or sludge synthesis than carbohydrates such as glucose (see literature review for carbohydrate values). While there was considerable variation in cell yield, the lower value was 35 percent, and the highest, 50 percent, it is emphasized that the coefficient of variance from the mean of 41.1 was only 8.6. When one compares this statistic to the coefficient of variance of 20.1 for the mean value for glucose of 61.9 percent (18), it is readily appreciated that the Y values for acetic acid were more closely grouped around the mean.

It has been proposed that differences in cell yield for heterogeneous microbial populations metabolizing an exogenous substrate under constant experimental conditions are due mainly to ecological variance of the population (49). The research data herein (Tables II, III, IV, and Figures 21-40) were obtained by employing a constant methodology and an identical experimental technique for each of the batch activated sludge-extended aeration systems and growth rate experiments. The only difference in the experiments was that introduced by variation in the species contained in the sewage seeds employed in each experiment. The fact that variations in cell yield were observed is attributable to differences in species predominance. Furthermore, the fact that the variance in cell yield for those randomly selected natural populations was considerably less when grown on acetic acid than when grown on glucose, would seem to be a manifestation of greater selectivity of acetic acid compared to glucose. That is, it would be expected that glucose could serve as growth substrate for a wider variety of microorganisms. Thus, because of greater diversity in predominating species in any

given experiment there would be a greater diversity in yield values.

Regarding the expected greater degree of species selectivity exerted by acetic acid, it is known that for microorganisms utilizing acetic acid as growth substrate, the inducement of the two adaptive enzymes of the glyoxalate cycle, malate synthatase and isocitratase, must take place (50). It is suggested that this rather specialized genetic capability is possessed by far fewer species than those capable of metabolizing simple carbohydrates such as the hexoses.

2. Growth Rate Experiments

As was observed from Figures 41-62, the major portion of growth rate data fit the hyperbolic function proposed by Monod for describing the relationship between specific growth rate (μ) and substrate concentration. Figures 63-69 show a representative set of optical density (logarithmic) vs. time curves of the type constructed for each growth experiment for determination of specific growth rate (μ). From Figures 63-69, as well as all of the other optical density (logarithmic) vs. time curves, it was observed and therefore necessarily concluded that exponential growth (described by the slope of a linear portion of a plot of optical density [logarithmic] vs. time on semi-logarithmic graph paper) occurred at substrate concentrations below maximum growth rate. These results corroborate the finding and are in accord with the hypotheses presented in a recent article by Gaudy, Obayashi, and Gaudy (31). They are contrary to Monod's empirical formula (equation 7) which indicates that exponential growth occurs only in the presence of excess substrate concentration.

It can be seen (Tables II, III, IV, and Figures 41-62) that an average maximum growth rate value of 0.705 hr^{-1} for heterogeneous

populations growing on acetic acid was obtained. This value is slightly higher than average μ_{max} values, e.g., 0.53 hr^{-1} reported for carbohydrate like glucose (51). It is suggested here that slightly higher values for maximum growth rate occurred because of the lower degree of diversity of species in the population due to the higher degree of selectivity of acetic acid. The mean value for the accompanying saturation constant (K_s) for acetic acid was 60 mg/l and is slightly lower than the range of 75-150 mg/l reported for glucose.

C. Endogenous Metabolism

It was observed in Tables V and VI and in Figures 1-20 that the total aerobic auto-oxidation of an accumulated sludge mass developed during the substrate removal phase by heterogeneous microorganisms of sewage origin growing on acetic acid as sole carbon source was definitely possible. These results are at variance with those reported by Symons and McKinney (35). They concluded that "total oxidation" of acetic acid is not possible, due to accumulation of a residual non-biodegradable organic material during the endogenous oxidation phase which they identified as polysaccharide. In a recent article, Obayashi and Gaudy (43) have shown that bacterial extracellular heteropolysaccharide does not constitute a biologically inert fraction.

The net accumulations of biological solids were shown in Tables V and VI and as a measure of total oxidizability values are expressed as percent theoretical COD and percent sludge synthesized. It was seen that in many of the batch activated sludge-extended aeration systems, values for "total oxidation" were much lower than values previously reported by earlier research workers (52)(40)(39).

It was seen (Figures 8-20, Tables III, V, VI) that during the

substrate removal phase, the cells did not synthesize large amounts of cellular carbohydrate as a primary endogenous storage product. The percent carbohydrate content of the cells remained relatively low and fairly constant through the entire period of endogenous metabolism. There was no buildup in carbohydrate content (percent) of the sludge during endogenous metabolism, offering further evidence that carbohydrate material is not biologically inert.

The primary cell storage component and endogenous carbon source synthesized by the bio-mass was protein (Tables II and III). Since protein constituted the energy "storage" product and endogenous substrate, the rapid utilization and extensive oxidation of proteinaceous molecules at the beginning of endogenous metabolism was observed (Figures 8-20). For many of the batch-activated sludge-extended aeration systems, a high degree of "total oxidation" of synthesized protein occurred during extended periods of endogenous respiration (Figures 8-20).

CHAPTER VII

SUMMARY AND CONCLUSIONS

A. Long-term Once-fed Batch Activated Sludge-Extended Aeration Studies Using Acclimated Natural Microbial Populations of Sewage Origin Growing on Acetic Acid as Sole Carbon Source

The present research investigation was a kinetic and metabolic study of acetic acid metabolism consisting of twenty long-term experiments covering both substrate removal and endogenous phases. Based upon this study, the following conclusions are drawn:

1. During the substrate removal phase, both substrate removal and sludge synthesis underwent a period of first-order increasing rate kinetics.

2. A mean cell yield for acetic acid (a two-carbon compound) of 41.1 percent was determined, and hence it is noted that it was lower than values reported for carbohydrates (hexoses).

3. Sludge yield can be considered constant for heterogeneous microorganisms during the entire course of autocatalytic growth and can be accurately determined by calculating the slope of the straight-line plot of biological solids vs. substrate (COD) removed.

4. Differences in cell yield values for heterogeneous populations grown under identical experimental conditions result from ecological variance of the populations. The degree of variance in cell yield

values is expected to be lower for substrates which are expected to exert a greater selective pressure on species variance. Thus, the values for Y are more closely grouped around the mean for acetic acid than for glucose.

5. The "total oxidation" of an accumulated sludge mass developed from the metabolism of acetic acid is definitely possible. Forty percent of the batch-activated sludge-extended aeration systems exhibited "total autodigestion."

6. Synthesis of protein was the primary cellular product and, during endogenous metabolism, served as primary endogenous carbon source.

B. Growth Kinetics

Growth rate experiments were conducted to determine the effect of substrate concentration on specific growth rate. Based upon this study, the following conclusions are drawn:

1. Exponential growth (as described by the slope of a linear portion of a plot of optical density [logarithmic] vs. time on semi-logarithmic paper) can exist at substrate levels below those needed to attain μ_{max}.

2. The hyperbolic function suggested by Monod for relating specific growth rate to substrate concentration adequately represents the relationship between exponential growth rate and initial substrate concentration.

CHAPTER VIII

SUGGESTIONS FOR FUTURE WORK

Further studies of the kinetic behavior and metabolic patterns of sludge oxidation during endogenous metabolism.

A basal theoretical premise of the "total oxidation" theory is that if total auto-oxidation occurs, then the accumulated amount of O_2 uptake should approximate the amount of biological organic matter available to the microorganisms, i.e., the theoretical O_2 demand is approximately equal to the theoretical COD. Confirmation of this theoretical premise can be obtained by operating a batch-activated sludge-extended aeration system and measuring oxygen uptake, biological solids, chemical oxygen demand, ammonia and nitrate nitrogen, and determinations of energy, materials, and nitrogen balances to ensure assessment of recovery of the carbon.

A positive result from this experiment should provide further proof as to the validity of the "total oxidation" theory, and should yield additional information about oxygen utilization and metabolic mechanisms during endogenous metabolism.

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