EFFECT OF SPECIFIC GROWTH RATE ON YIELD OF *ESCHERICHIA INTERMEDIA* GROWING IN BOTH CONTINUOUS AND BATCH SYSTEMS

By

CROSBY WILSON JONES, JR.

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Thesis Approved:

Elizabeth T. Gaudy
Thesis Adviser

Dean of the Graduate College

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CHAPTER I

LITERATURE REVIEW

Most discussions of bacterial yield and maintenance energy begin by citing the early work of Jacques Monod. The present review will be no different. Although other investigators preceded him in the study of various aspects of this concept, Monod (1942) was the first to develop the concept of substrate-based yields which later provided a basis for the maintenance concept. He showed a simple relationship between growth and utilization of substrate. From this he developed the all important yield constant. This growth constant is defined over any finite period of growth as

$$\frac{\text{dry weight of bacteria formed}}{\text{weight of substrate used}} = Y. \qquad (1)$$

Most yield studies have used this basis for yield calculation. However, Bauchop and Elsden (1960) introduced the concept of yield based on amount of ATP obtained from the degradation of the energy source. This method of yield calculation has proven useful with anaerobic bacteria but has not been perfected for aerobic bacteria because of a lack of information regarding the exact amounts of energy formed for any particular substrate. Both methods of measuring yield will undoubtedly contribute to the present knowledge of interrelationships between growth, yield and maintenence

Monod (1942) also introduced, in the form of an equation, a relationship between the specific growth rate (μ) and the concentration

of an essential growth substrate (S). The specific growth rate is defined as the rate of increase per unit of organism concentration $\left(\frac{dx}{dt} \cdot \frac{1}{x}\right)$. It was shown to be proportional to substrate concentration to a limiting saturation value as described by the equation

$$\mu = \mu_m\left(\frac{S}{K_s + S}\right), \qquad (2)$$

where μ_m is the maximum value that μ can reach and K_s is the saturation constant. μ_m is that value at which further increases in initial substrate concentration cause no further increase in μ. K_s is numerically equal to the substrate concentration at which $\mu = \frac{1}{2}\mu_m$. Both μ_m and K_s are constants under a given set of experimental conditions for a particular strain of bacteria. This equation, known as the Monod equation, has proven extremely valuable as an aid to the understanding of the kinetics of microbial growth. It has been shown to fit a large amount of experimental data. The results of a series of experiments designed to test the validity of the Monod equation have been presented by Gaudy, et al. (1971). No "perfect" mathematical representation of all bacterial growth has been found nor is there likely to be such a treatment.

The continuous culture technique developed by Monod (1950), Novick and Szilard (1950) and Herbert, et al. (1956) has increased the number of mathematical treatments describing growth kinetics. Applied to continuous culture, the Monod equation has again proven to be extremely useful. However, two faults become evident when applying the equation to this type of system. The first of these is the insensitivity of μ to small changes in S during the exponential growth phase where S is much higher than K_s. This has been called slippage by Gaudy, et al.

(1971). They have proposed that the symbol S_0 be used in the Monod equation instead of S to represent initial substrate. Secondly, Mateles, et al. (1965) showed that during transient operation of a continuous culture, Monod's equation could not be used to relate specific growth rates and concentrations of limiting nutrient. Although it should not be used in critical measurements of the transient, it can be useful as a rough approximation of this growth. The Monod equation has proven its value in numerous experiments and is the expression of choice in the present study.

Since the inception of continuous culture in the early 1950's, this technique has been utilized in many studies involving bacterial physiology and genetics. Monod and Cohn (1952) used the technique to study adaptive enzymes while Novick and Szilard (1952) were using it to study bacterial genetics and physiology. Recently, Palumbo and Witter (1969) and Mennett and Nakayama (1971) have used the technique for studying the influence of temperature on microbial growth. A myriad of uses for the technique has been developed. All of these uses take advantage of the inherent capability of the system to allow balanced growth of microorganisms over a wide range of specific growth rates. The only change in the organisms' environment occurs when the rate is changed. This results in a change in the concentration of the limiting nutrient which rapidly results in a change in the steady state of the culture. The complications introduced by using batch cultures are avoided. Theoretical treatments as well as experimental studies by Monod (1950), Herbert, et al. (1956) and Novick and Szilard (1950) have laid the foundations for use of the system. Probably the

most obvious advantage of a continuous flow system other than the balanced environment is the operator's ability to hydraulically control the specific growth rate by simply controlling the dilution rate. This results in a system whereby responses to specific growth rate may be measured under the same set of conditions.

The continuous flow technique provides a very convenient means of establishing yield (Y) for a range of specific growth rates. Using the chemical oxygen demand (COD) method for measuring substrate utilization (APHA, 1971), the yield can easily be calculated at any specific growth rate. This is becoming the preferred method because of its simplicity and accuracy. In addition, all substrate is measured regardless of whether it is in the form of an intermediate or present as the original substrate. Yields based on change in COD are consequently more accurate than yields based on the removal of the original substrate alone.

The yield for any particular pure culture is a constant for a specific set of conditions. Any change in the environment of the culture may also have effects on the yield. To illustrate, Mennett and Nakayama (1971) have shown with Pseudomonas fluorescens that the yield is dependent upon temperature of incubation. They obtained a progressively higher yield as the temperature was raised from 20°C to 30°C. At 35°C, the yield began to decrease. Ng, et al. (1962) also showed a temperature effect with Escherichia coli using a batch culture instead of a continuous flow system.

It is interesting to note that wherever yield is reported to be affected by some physical parameter, the specific growth rate has also been affected. This suggests a correlation between the two parameters. Monod (1942) presented early negative evidence when he demonstrated

with E. coli that the yield did not seem to be affected by specific growth rate. He limited the rate of growth by limiting aeration which doubled the time to achieve maximal density but left the yield unchanged. He obtained yields of about 0.23 for E. coli. These are quite low and Schulze and Lipe (1964) have postulated that the nutrient medium used by Monod was incomplete and there may have been other limiting factors controlling the experiments.

Bauchop and Elsden (1960) have presented evidence that Y_{ATP} is constant and has a value approximating 10.5 grams per mole of ATP. Many other workers have verified their results using a variety of genera. DeVries and Stouthamer (1970) have used Bifidobacterium bifidum, Twarog and Wolfe (1963) Clostridium tetanomorphum and Oxenburgh and Snoswell (1956) Lactobacillus plantarum. These examples typify the research being done in this area. Many other sources can be cited, all of which indicate a constant yield based on ATP formation. The data in all of these experiments were based on batch yields and were performed using anaerobic conditions. The majority of these experiments have neglected the interrelationship between specific growth rate and yield although such a relationship has been shown in a number of experiments. Seemingly, the results obtained from batch experiments would be more valuable if specific growth rates were presented with the data. Where these rates have been reported, an important concept has evolved. That is, as the specific growth rate increases, cell yield also increases. The reverse situation is also true (i.e., a decreased rate results in a decreased yield). This effect has been recognized in those experiments in which the specific growth rate was measured. Postgate and Hunter (1962) showed this effect with Aerobacter aerogenes using carbon,

nitrogen, phosphorus, and magnesium as limiting nutrients in several series of experiments. Herbert (1958) showed a similar effect using A. aerogenes and Torula utilis. Schulze and Lipe (1964) using E. coli performed the same type of experiment. They used glucose as the limiting carbon and energy source and were able to show that at a μ of 0.06 hr^{-1} a yield of 0.44 mg/mg was obtained. Conversely, at a μ of 0.66 hr^{-1} the value obtained for the yield was 0.54 mg/mg. From these results, it appears that at high specific growth rates substrate is more efficiently converted to cell material. Very little evidence has been presented to refute this common observation in pure culture. Senez (1962) using A. aerogenes and Harrison and Loveless (1971) using E. coli both showed an independence of cell yield to changes in growth rate. Y_{ATP} was used in the former and substrate yield in the latter. Interestingly, both used temperature as the limiting parameter. Using temperature instead of energy as a limiting factor may affect the physiology of the bacteria differently than energy limitation.

While substrate-based cell yields are an important parameter in the study of bacterial physiology, they may be even more important when applied to industrial production or waste purification. Both applications are important but the latter has in recent years been a focal point of interest. The microbiological and waste purification (bioengineering) disciplines are closely related in many aspects. Consequently, research in both areas has been pooled together and the result has been principles derived from two points of view. A major difference in the approach of the two areas toward research is in the methods by which microorganisms are studied. Since the pure culture technique was developed, microbiologists have used it almost exclusively in all research. Except

for microbial ecologists, very few microbiologists have been concerned with the interrelationships between microorganisms in mixed cultures. Conversely, bioengineers have seldom worked with pure cultures, their work being almost exclusively with the mixed flora necessary for the microbial degradation of wastes. Data from both areas of research have resulted in a number of observations which can be compared. Some of these are the same for both pure cultures and mixed populations while others are different. A case in point is the discussion of the variance in cell yields with changing growth rates. Sherrard, et al. (1973) have shown a dependence of the yield upon the specific growth rate for a mixed culture in a cell recycle system. This system is familiar to all bioengineers and involves recycling a portion of the cells to the primary reactor where the incoming substrate is degraded. The mathematical description of this process has been given an excellent treatment by Gaudy and Gaudy (1971). Although this system differs from both the continuous once-through system and the batch system, the relationship between growth rate and yield remains the same. Other investigators using the same type of system have noted the same effect. Hence, comparison of data between the two disciplines should enhance the research in both fields.

The present discussion has centered on the relationship between growth rate and cell yield. This relationship has been shown in batch systems, once-through chemostat systems and cell recycle systems. Both microbiologists and bioengineers have noted the same effect. The explanation for this phenomenon has been formulated around the concepts of endogenous metabolism and maintenance. Monod (1942) has postulated that theoretically a minimum substrate concentration should exist which would

allow only survival or maintenance of the cells. His attempts to measure this quantity were unsuccessful and as a result many subsequent workers have assumed that maintenance energy does not exist. However, those researchers who have since made attempts to measure this energy have presented evidence for its existence. Dawes and Ribbons (1964) have reviewed the aspects of endogenous metabolism and defined it as "the total metabolic reactions that occur within the living cell when it is held in the absence of compounds or elements which may serve as specific exogenous substrates" (pg. 126). In the absence of an exogenous substrate an endogenous substrate must be utilized to provide energy for maintenance. When the endogenous substrate is exhausted an exogenous energy source must be present or the cell dies. This, in essence, is the concept of maintenance energy. McGrew and Mallette (1961) recognizing the need for metabolic energy to meet the demand of chemical and physical wear and tear have defined maintenance energy simply as that amount of energy required to maintain the status quo. Cellular processes visualized as requiring this maintenance energy are those involving resynthesis of unstable macromolecules, osmotic regulation, maintenance of a constant intracellular pH and motility.

The correlation between growth rate and cell yield is apparently a manifestation of the maintenance energy. Stouthamer and Bettenhaussen (1973) have indicated that Y_{ATP} is dependent upon the carbon content of the cells, the growth rate and the maintenance coefficient. To explain the high yields obtained when cells are grown at high growth rates, they present evidence to show that the relative proportion of energy needed for maintenance is less than at lower growth rates. Consequently, higher yields are obtained at high growth rates.

The maintenance coefficient is defined as the substrate requirement for energy of maintenance per unit amount of organism per unit time. Several equations and methods have been proposed for measuring this coefficient. Pirt (1965) and van Uden (1967) have presented their own descriptive equations as well as reviewing earlier treatments by Marr et al. (1963) and Schulze and Lipe (1964). Marr plotted the reciprocal of the steady-state turbidity against the reciprocal of the dilution rate to get a linear function with an ordinate intercept of $\frac{1}{X_{max}}$ and a slope of $\frac{a}{X_{max}}$. This function is described by the equation $\frac{1}{X} = \left(\frac{a}{X_{max}} \cdot \frac{1}{D}\right) + \frac{1}{X_{max}}$ where X is the concentration of cells, D is the dilution rate, X_{max} is the concentration of bacteria that would be supported if the specific maintenance rate was zero and a is the specific maintenance rate. Pirt's equation for maintenance, $\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_G}$, describes a straight line function when $\frac{1}{Y}$ versus $\frac{1}{\mu}$ is plotted. The maintenance coefficient m and yield corrected for maintenance Y_G are assumed constant. This method has been used frequently to determine the maintenance coefficient but Marr's method has also been used in some situations. The mathematical derivations of these equations are beyond the scope of this review. None of the equations nor any of their modifications have been shown to be most universally applicable. Different situations arise in which one method of calculation may prove to be more satisfactory than another. At the present, the most exact measurement of maintenance is by a combination of methods.

Specific values for the maintenance coefficient have been reported for both Aerobacter cloacae and E. coli. Pirt (1965) using A. cloacae calculated a maintenance coefficient of 0.094 g substrate/g dry wt/hr

when the substrate was glucose under aerobic conditions. Using the same organism under anaerobic conditions, he obtained a maintenance coefficient of 0.473. This illustrates the effect of culture conditions on maintenance energy. Using E. coli, Schulze and Lipe calculated a maintenance coefficient of 0.055. This was at a temperature of 30°C and under aerobic conditions. Pirt (1965) using Marr's data with E. coli at 30°C has calculated a maintenance coefficient of 0.070 which approximates the value obtained by Schulze and Lipe (1964). These values for the maintenance coefficient illustrate an important fact concerning the maintenance theory. Direct attempts at measuring this value have proven extremely difficult. An examination of the calculated values shows why. Direct measurement of a value on the order of 0.05 - 0.07, is a difficult task to perform accurately without better techniques than are available presently. Monod (1942) was unable to detect any maintenance energy using E. coli although he postulated that such energy should be present. His techniques were not sensitive enough to measure the quantity. Although the value is low, the ultimate result on cell yield can be very appreciable. This is especially true at low growth rates where a proportionally larger amount of available energy must be used for cell maintenance. Thus, the theory of maintenance has been used to explain the dependence of cell yield upon growth rate.

Until better methods have been derived for the demonstration of maintenance, the theory must remain as the probable explanation for the yield-growth rate relationship. McGrew and Mallette (1962) have made direct attempts at measuring the maintenance coefficient in E. coli. Their method involved adding glucose to the culture until a threshold level was obtained so that a particular turbidity was maintained which

neither increased nor decreased. It was concluded that this level of glucose was specifically utilized for maintenance without growth. An amount of 0.1 mg of glucose was needed to maintain 1.5 mg of cells. Converting this value to the standard maintenance notation (i.e., gm substrate/gm dry wt/hr) a value of 0.067 is obtained. This closely correlates with the values derived by Schulze and Lipe (1964) and Pirt (1965) for E. coli in which values of 0.055 and 0.070, respectively, were obtained. McGrew and Mallette used a temperature of 37°C compared to the 30°C used in the other two experiments. It appears this difference in temperature did not alter the maintenance energy. However, it should be noted that different strains were used in the experiments. Mennett and Nakayama (1971), Palumbo and Witten (1969) and Marr (1963) have all shown a temperature effect on the maintenance coefficient.

The problems in measuring the maintenance coefficient have been elaborated on by McGrew and Mallette (1962) and Mallette (1963). They point out that since most of the earlier workers used small inocula and grew the cultures for long periods of time, the ratio of the weight of energy source initially present to the weight of cells at the experiment's end was relatively high. Therefore, they concluded that low threshold levels would be difficult to determine by extrapolations. Their method involved a much lower ratio of glucose to cell weight. Marr, et al. (1963) have pointed out that this method restricts the growth rate of the cells and thus probably alters the maintenance coefficient. Also, growth was followed by turbidity which may also result in some difficulties in distinguishing between dead and living cells.

Stouthamer and Bettenhaussen (1973) have concluded that as far as Y_{ATP} is concerned, the specific growth rate and the maintenance energy are of the utmost importance in calculating these yields. Although accurate measurements of maintenance present many problems, indirect evidence as well as some direct evidende have indicated that an appreciable portion of the energy source must be utilized for maintenance especially at low growth rates. Dawes and Ribbons (1964) have voiced concern over maintenance evaluation determined from experiments using a single compound as sole energy and carbon source. They feel this technique does not present a convincing argument that energy not used for growth is specifically diverted to maintenance. To prevent complications involving use of the energy source as carbon source, they suggest using microorganisms which do not incorporate their energy source into cell substance. Seemingly, this would involve even more complications and result in the same problem of determining whether energy not used for growth is directed to maintenance. Neither substrate-based yields nor Y_{ATP} have conclusively solved the problem. Hence, the two methods of measuring yields continue in common use. Certainly, the limiting energy source and carbon source whether they are the same or not should be reported in all experimental results. In addition, environmental conditions must be known and stated. Temperature and aeration effects on maintenance energy have been cited. pH may also be expected to affect the maintenance coefficient. Stouthamer and Bettenhaussen (1973) have also reported that the composition of the growth medium has a strong influence on the maintenance coefficient. All of these factors affect the values obtained for maintenance. As a sequitor it is expected that yields will also be affected. However, this has been shown not to

be the case in at least one instance reported by Stouthamer and Bettenhaussen (1973) for Y_{ATP}. They showed with A. aerogenes that in a medium with high NH_4Cl content the maintenance coefficient increased without a change in Y_{ATP}. Decreasing the NH_4Cl content resulted in a decrease of the maintenance coefficient but with no effect on Y_{ATP}. This observation may be interpreted as a maintenance requirement for maintaining the correct ionic composition of the cells but it does not explain the constant Y_{ATP}. Disregarding faulty experimental technique, it must be assumed that either the cells make more efficient use of their energy at high NH_4Cl levels or some factor other than maintenance is operative in this system. Senez (1962) has presented evidence of a determining factor in yield studies which differs from the maintenance theory. Basically this theory predicts a coupled relationship between growth and energy production. When optimum conditions are present this coupling is tight and yield is for the most part based on growth rate and the maintenance coefficient. However, under unfavorable conditions this coupling is not as tight and consequently some of the energy is wasted and optimum growth does not occur. Senez has shown this phenomenon using high temperatures. Belaich, et al., (1972) and Lazdunski and Belaich (1972) have demonstrated the uncoupling phenomenon with Zymomonas mobilis upon pantothenate starvation. Uncoupling has also been implicated during the transition periods between specific growth rates in chemostat cultures (Tempest, et al., 1967).

At the optimum conditions for a given strain of bacteria, cell yield is maximized with respect to inherent genetic specification, growth rate, coupling and maintenance. If any of these parameters are altered, cell yield is very likely to change also. The genetic

inheritance and coupling are less likely to change unless very adverse conditions or mutation occurs. The specific growth rate and maintenance coefficients are the two most easily changed parameters and would be expected to affect yield more readily. The theory of maintenance has recognized this fact and most changes in yield have been attributed to either a change in specific growth rate or maintenance energy.

A recent study by Gaudy and Srinivasaraghavan (1974) with a glucose-limited mixed culture has interestingly both supported and opposed the maintenance theory. They employed a cell-recycle system, a once-through system and a batch system in these experiments. It was discovered that growth in a recycle system at very low specific growth rates resulted in yields that increased when the specific growth rate was increased. However, when the recycle system was converted to a once-through system at a higher specific growth rate, the yield remained the same. Furthermore, when inocula were taken from the once-through systems and grown in batch, the yield still retained the original cell-recycle yield value. Theoretically, maintenance should be at its minimum at μ_{max}. Batch reactors in which substrate is in excess should allow maximum yields because maximum specific growth rate and minimum maintenance are expressed in such a system. The maintenance theory is upheld in the cell recycle data but opposed in the once-through and batch systems where yields were expected to rise as a result of the higher specific growth rate and minimum maintenance in these systems. It was theorized that a selection of species occurred which resulted in a low yield population replacing the higher yield species. Gaudy, et al. (1967) and Ramanathan and Gaudy (1969) have shown that growth at

increased specific growth rates tends to select cells with a higher μ_{max}. Thus selection is not unexpected in a mixed culture.

The present investigation was undertaken to clarify these results. This involved using a pure culture so that selection of species could be avoided. The results of such a study can provide clarifying insights to the concept of maintenance energy.

CHAPTER II

MATERIALS AND METHODS

Test Organism

The organism used in this study was isolated from raw sewage in Stillwater, Oklahoma. It was selected from an eosin methylene blue lactose agar plate on which it appeared as a flat colony with a green sheen. It was subsequently identified as Escherichia intermedia by a series of biochemical tests.

Identification of the Test Organism

The Seventh Edition of Bergey's Manual (1957) was consulted to select the various differential media used in identifying the organism. Those used included glucose, lactose, mannitol, sucrose, glycerol, nitrate, Koser's citrate, urea, and tryptone broths. Bromcresol purple was used as the pH indicator for fermentation tests. All except the urea and citrate broths included Durham tubes to detect gas formation. In addition, litmus milk, Kligler's iron agar slants and methyl red-Voges Proskauer (MRVP) media were used. All incubations were at 37°C. Readings were taken in all cases at 24 and 48 hours, except the litmus milk, nitrate broth, tryptone broth and the methyl red portion of the MRVP test. The litmus milk was read at 1, 3 and 7 days. The nitrate broth and the MRVP broth for testing methyl red reaction were completed at 5 days and the tryptone broth was tested for indole production at 24

hours. These results were supplemented with a Gram stain, wet mount and negative stains using India ink and nigrosin. Bergey's Manual was consulted for the final identification.

Media

Minimal medium was used throughout this study in both the continuous flow and the batch systems. Composition of the medium per liter of solution was: $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.5 mg; $CaCl_2 \cdot 2H_2O$, 7.5 mg; KH_2PO_4, 3 g; Na_2HPO_4, 6 g; yeast extract (Difco), 1 mg; and tap water, 100 ml. For the continuous flow studies glucose was used in a limiting concentration of 1000 mg/l. For batch studies 1000 mg/l of glucose was also used as well as lower concentrations in certain experiments. All ingredients of the medium were autoclaved separately, cooled, and mixed aseptically. Medium containing extra $MgSO_4 \cdot 7H_2O$, 0.2 g, and extra $(NH_4)_2SO_4$, 0.75 g, was utilized in one experiment.

Tryptic soy agar plates and slants and eosin methylene blue lactose agar plates were prepared by rehydrating the powdered media (Difco) with distilled water. The plates, containing 30 ml each of the medium, were dried at 37°C before use.

For the phage experiments, phage plates were made using per liter of distilled water: nutrient broth, 8 gm; NaCl, 5 gm; and agar, 11 gm. Each plate was poured with 40 ml of medium. The plates were dried at 37°C for 24 hours before use.

Top agar was made by using per liter of distilled water: nutrient broth, 8 gm; NaCl, 5 gm; and agar, 6.5 gm. This medium was distributed in 3 ml amounts into small test tubes fitted with plastic tops.

Phage broth, consisting of 8 grams of nutrient broth and 5 grams of NaCl per liter of distilled water, was dispensed into 6 oz. medicine bottles.

Cultivation of the Test Organism

Stock cultures of E. intermedia were transferred to new TSA slants once a month. The inoculated slants were incubated at 37°C for 12-18 hours and stored at 4°C.

For most experiments cells used as inoculum were grown in minimal medium overnight at 37°C on a reciprocal shaker. For the phage studies, the cells were grown in 5 ml of phage broth overnight on a reciprocal shaker at 37°C.

Yield Measurements

Yield measurements were obtained from both continuous flow and batch systems. Samples of 20 ml were taken for yield analysis at the appropriate times from the continuous system or at the end of the substrate removal phase in batch systems. The batch reactors contained 60 ml of medium each. Growth in both systems was followed by spectrophotometry using a Coleman Junior Spectrophotometer, Model D at 540 nm in either 18 mm test tubes or sidearm flasks. The cells were filtered through tared membrane filters (pore size 0.45μm) for determination of cell concentration (mg/l dry wt). The filtrate was removed into a prepared chemical oxygen demand (COD) flask and analyzed for total organic matter by oxidation with sulfuric acid-potassium dichromate (COD test, APHA, 1971). The cells were washed twice on the filter with distilled water and the filters were dried to a constant weight in an oven overnight

at 105°C. The dry weight of the sample was determined by weighing the dried filter paper and sample on an analytical balance. Yield was determined by dividing the dry weight of the sample by the change in the chemical oxygen demand (ΔCOD). The ΔCOD represents the difference between the initial feed COD and the sample filtrate COD.

Continuous Flow Studies

A 2.5-liter chemostat with a working volume of one liter was used throughout the continuous flow studies. It was fitted with a cap with two air inlets, two air outlets and one feed inlet. The overflow outlet was positioned on the side of the chemostat tube. Two carborundum air spargers were fitted to the cap by rubber tubing for aeration. An air jet was used as a source of aeration. Contamination was prevented by passing the air through a cotton-filled 500 ml Erlenmeyer flask which had previously been sterilized. In addition, an air filter apparatus of 0.45μm pore size was included in the system.

The feed reservoir was a 20-liter carboy in most of the experiments. A 10-liter carboy was used in several experiments. The medium was pumped from the reservoir and into the chemostat by a Simplex Milroyal model D controlled volume pump. A Sigmamotor peristaltic pump was used in several of the experiments. Both pumps were fitted with tubing having 1/4" diameters and a 1/8" bore. The Milroyal pump was equipped with Tygon tubing and the Sigmamotor with vacuum rubber tubing. The whole chemostat system was autoclaved except the Milroyal pump which was flushed with chlorine.

Aeration was gauged by a Gelman air flow meter and temperature was

held constant by placing the chemostat in a constant-temperature water bath.

Continuous flow studies were performed at either 30°C or 25°C with an aeration of 3 liters per minute which was sufficient to provide both aeration and adequate mixing.

Cells were inoculated into 25 ml of minimal medium and put on a reciprocal shaker at either 25°C or 30°C for 12 hours. These cells were aseptically introduced into the chemostat and the pump was activated. One liter of sterile medium was allowed to fill the working volume of the chemostat. Aeration was begun and the pump was shut down until, by visual examination, the culture appeared to be growing adequately. The dilution rates used in these studies were 1/6 hr^{-1} and 1/24 hr^{-1}. These rates were achieved by varying the medium delivery from the pump. The dilution rate was determined by the flow of incoming medium divided by the working volume of the chemostat. The pumps were calibrated as described later.

Growth was ascertained by taking samples from the outflow and measuring absorbance on the spectrophotometer. A steady state was assumed when transmittance did not vary by more than one unit over 24 hours. When the dilution rate was changed, absorbance was again followed until a new steady state transmittance was reached. This usually took 24 hours but samples for dry weight were not taken until 48 hours had elapsed in most cases. In those instances where samples were taken at less than 48 hours, careful attention was paid to the yields obtained, making sure they represented steady state values and not transient values. Absorbancies for the transition from 1/24 to 1/6 hr^{-1} reflect the temporary use of a spectrophotometer defective in calibration.

Experiments were initiated to determine the yields of E. intermedia in a chemostat at a dilution rate of 1/6 hr^{-1} as compared to yields at 1/24 hr^{-1}. A number of samples for yield determination were taken at each steady state. The transient going from 1/6 hr^{-1} to 1/24 hr^{-1} and vice versa was monitored by spectrophotometry. During operation at both dilution rates, small amounts of chemostat effluent were employed as inocula for batch growth studies using a series of concentrations of glucose in the same medium being fed to the chemostat.

Pump Calibration

Two different types of pumps were used in these studies. The Milroyal pump consisted of a drive unit, plunger and displacement chamber in which the plunger reciprocated. The pump delivered a controlled volume of liquid with each discharge stroke. Pump delivery was adjusted by changing the plunger stroke manually.

The Sigmamotor pump worked by peristaltic action. Pump delivery was changed by adjusting to the desired per cent of full speed.

For calibration, each pump was fitted with its tubing and prepared just as in the actual experiments. A flask was placed at the same level as the chemostat lid and pump delivery was collected here. The pumps were adjusted to a certain speed which was recorded and, after a specific time interval (in most cases 1 minute), the delivered water was measured in a graduated cylinder. This was converted into delivery expressed as ml/hr. A series of pump adjustments were made to prepare a calibration curve for each pump. These are shown in the results section.

Batch Studies

Batch experiments were performed simultaneously with the continuous flow experiments using inocula taken from the chemostat. One series was performed using 1/6 hr^{-1} inocula and the other with 1/24 hr^{-1} inocula. The medium used in the batch experiments was the same as that used in the chemostat except where the growth constants K_s and μ_m were determined. These constants were calculated from various μ values obtained at various substrate concentrations. A straight-line form of the Monod equation, $1/\mu = (1/\mu_m) + K_s/\mu_m S$ (Monod, 1942) was used to determine μ_m and K_s. Growth was followed on the Coleman Junior Spectrophotometer at 540nm. Specific growth rate was determined first by plotting absorbance versus time on semi-logarithmic paper. μ was calculated from this graph using the equation $\mu = 0.693/t_d$, where t_d is the doubling time. This equation is derived from $dx/dt = \mu x$ which is in the form of a first order differential equation; x represents the biological solids. An integrated form of this equation is $\ln X_t - \ln X_0 = \mu t$. The doubling time, t_d, is determined from the semi-log plot and substituted into this equation. μ is then defined as $\mu = \ln 2/t_d = 0.693/t_d$. The batch experiments were performed in 250 ml sidearm flasks using 60 ml of medium. These were incubated at the appropriate temperature in a Warner-Chilcott shaking water bath at 105 cycles/minute with a 1" stroke length. Approximately 1-3 ml of inoculum was aseptically introduced to each batch flask. This was sufficient to raise the initial optical density to 0.02. Dry weight determinations on the inocula indicated an introduction of approximately 20 mg/l of cells. This was corrected for in final yield determinations.

When the substrate removal phase was completed, yield determinations were performed as described in the yield measurement section of this chapter.

In one series of experiments, inocula were taken from batch flasks which had just finished the substrate removal phase and introduced into a new set of batch flasks containing 60 ml of medium to determine the effect of sequential batch growth on the cell yield. All other conditions and measurements were performed as described above.

Two series of experiments were performed in batch using inocula not previously passed through the chemostat. The inocula in both cases was taken from twelve hour batch cultures inoculated directly from the slant. Each of the batch reactors in these experiments contained 25 ml of medium. All other analyses and experimental conditions were as described previously.

The first of these batch experiments was performed to determine if there was a difference in specific growth rate or yield between two morphologically distinct colony types, one of which appeared to have arisen during the course of the chemostat studies. A stock culture was kept of each colony type and utilized in this series of experiments. Biochemical tests were performed prior to the batch studies for reassurance that the two cultures were both E. intermedia.

The second series of experiments not utilizing inocula from the chemostat involved testing the culture for yield responses to extra magnesium and ammonium sulfate. Also a comparison between washing the cells with distilled water and buffer was performed in this series of experiments. The buffer used was 0.03 M sodium phosphate (pH 7.1). The medium utilized in this series of experiments contained 0.75 g/l

of $(NH_4)_2SO_4$ and 0.2 g/l of $MgSO_4 \cdot 7H_2O$ with all other components and concentrations remaining the same as in previous experiments. These batch flasks were inoculated and placed on the shaker at 30°C. After the substrate removal phase, each 25 ml sample was removed for yield determinations.

Phage Studies

Phage experiments were performed in an attempt to distinguish between the two morphologically distinct colony types. The two types were labelled throughout these experiments as R and S. The R colonies were a normal flat type on EMB lactose agar plates. The S colonies were raised and mucoid on EMB lactose agar. Both colony types were very nearly identical on TSA plates although they could be distinguished within 12-24 hours after plating.

The phages used in these experiments were isolated from raw sewage. Two drops of chloroform were added to 5 ml of raw sewage to destroy all bacteria. The chloroform was subsequently removed by aeration. Small test tubes containing melted sterile phage top agar in 3 ml aliquots were kept in a 47°C water bath and 0.1 ml of chloroform-treated sewage was added to each of two tubes of the top agar. A loopful of an 8-hour culture of R grown in phage broth on a 37°C shaker was added to the melted top agar and sewage. This procedure was repeated with the S culture. After mixing, the tubes of top agar were poured on separate phage plates and allowed to solidify. The plates were inverted and incubated at 37°C for 24 hours. The plates were then examined for plaques. Using a short needle, differing plaques were picked and shaken into tubes of sterile phage broth. Two drops of chloroform were added

to each tube and then removed by aeration. Each of these tubes was labelled and replated at different dilutions so as to result in 100-300 plaques per plate. Typical plaques were picked and shaken into another tube of sterile phage broth. These were replated two more times to assure the purity of each phage culture. Each isolate was labelled by designating whether it was isolated on R or S.

Plate stocks of each phage were made from these pure cultures. Each culture was replated twice using 0.1 ml of undiluted phage suspension and a drop of an 8-hour culture of the appropriate bacteria mixed together in 3 ml of phage top agar. Each pair of plates was incubated for 12 hours at 37°C. After incubation, 5 ml of phage broth was poured onto each plate and allowed to soak for 30 minutes. After soaking, a syringe was used to remove the broth. Between 8-10 ml of broth were obtained for each pair of plates. This was centrifuged to remove unlysed bacterial cells and then passed through a membrane filter (0.45μm pore size) to a sterile bottle. These plate stocks were kept at 4°C in a walk-in refrigerator.

Dilutions of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} were made in phage broth using each of the plate stocks. These were plated with the appropriate bacterial culture to obtain a rough estimate of the stock titers.

A countable titer of each phage plate stock was replated in triplicate with both the R and S bacterial cultures. Thus, three platings were made with R and three were made with S for each phage. Accurate titers as well as sensitivities of S and R cultures to the different phages were determined from this procedure. All incubations were at 37°C and plates were examined for plaques in 24 hours.

CHAPTER III

EXPERIMENTAL RESULTS

Identification of the Test Organism

The organism used in these studies was isolated from municipal sewage and subjected to a number of differential tests for subsequent identification of the genus and species. The cells were Gram negative, short rods showing motility in nutrient broth. No readily apparent capsule could be seen. Growth on TSA plates yielded small colorless colonies with entire margins. Upon EMB-lactose plates the colonies gave a characteristic green sheen and were small, flat and somewhat rough with entire margins. Using these morphological characteristics and the results presented in Table I, the organism was identified after consulting Bergey's Manual as E. intermedia. It was identified as a member of the genus Escherichia primarily because of its positive indole reaction, positive methyl red test, nitrate reduction to nitrite and acid and gas in lactose broth within 48 hours at 37°C. Its green sheen on EMB lactose is also characteristic of the genus Escherichia. The designation of the species as intermedia was based upon the use of citrate as sole carbon source. E. intermedia is the only member of this genus which does so. All other tests were utilized as supplementary identification measures.

TABLE I

IDENTIFICATION OF THE TEST ORGANISM

Test	Results
Glucose utilization	Acid, no gas (48 hours)
Lactose utilization	Acid and gas (48 hours)
Mannitol utilization	Acid, no gas
Sucrose utilization	Acid, no gas
Glycerol utilization	Growth, neutral pH
Citrate as sole carbon source	Positive
Nitrate reduction	Positive to nitrite
Urease production	Negative
Indole production	Positive
Methyl Red	Positive
Voges-Proskauer	Negative
Kligler's iron agar slants	No H_2S, acid throughout
Litmus milk	Acid curd and some reduction
Gram stain	Negative
Cell morphology	Short single rods
Capsule stain	Very small capsule

Pump Calibrations

The two pumps used in the continuous flow studies were calibrated in the manner described earlier. The Sigmamotor pump delivered medium at a more efficient rate than the Milroyal pump for comparable percentages of capacity. However, the Milroyal pump delivered feed at a more constant rate than did the Sigmamotor which tended to fluctuate slightly in delivery. The Sigmamotor pump proved more convenient because sterilization of the tubing could be performed with an autoclave rather than by flushing chlorine through the lines as was required with the Milroyal. The Sigmamotor pump was also smaller and easier to handle. The steadiness of medium delivery is shown in Figures 1 and 2. Straight line plots were obtained with both pumps. These results allowed the confident use of either pump for accurate delivery of medium into the continuous flow system thereby establishing a constant dilution rate. Calculations of the dilution rates are given in Tables II and III for both pumps.

Continuous Flow Studies

The continuous flow experiments involved the use of the chemostat described earlier. Contamination of the system was a problem at various times during the course of these experiments. Frequent microscopic examination as well as sample plating were used to check the purity of the culture. When contamination occurred, the entire chemostat was dismantled, cleaned and placed in the autoclave for up to an hour to insure sterilization. Sources of contaminant entry were minimized by several small adjustments of the system such as lengthening the overflow tubing or placing cotton in all air outlets.

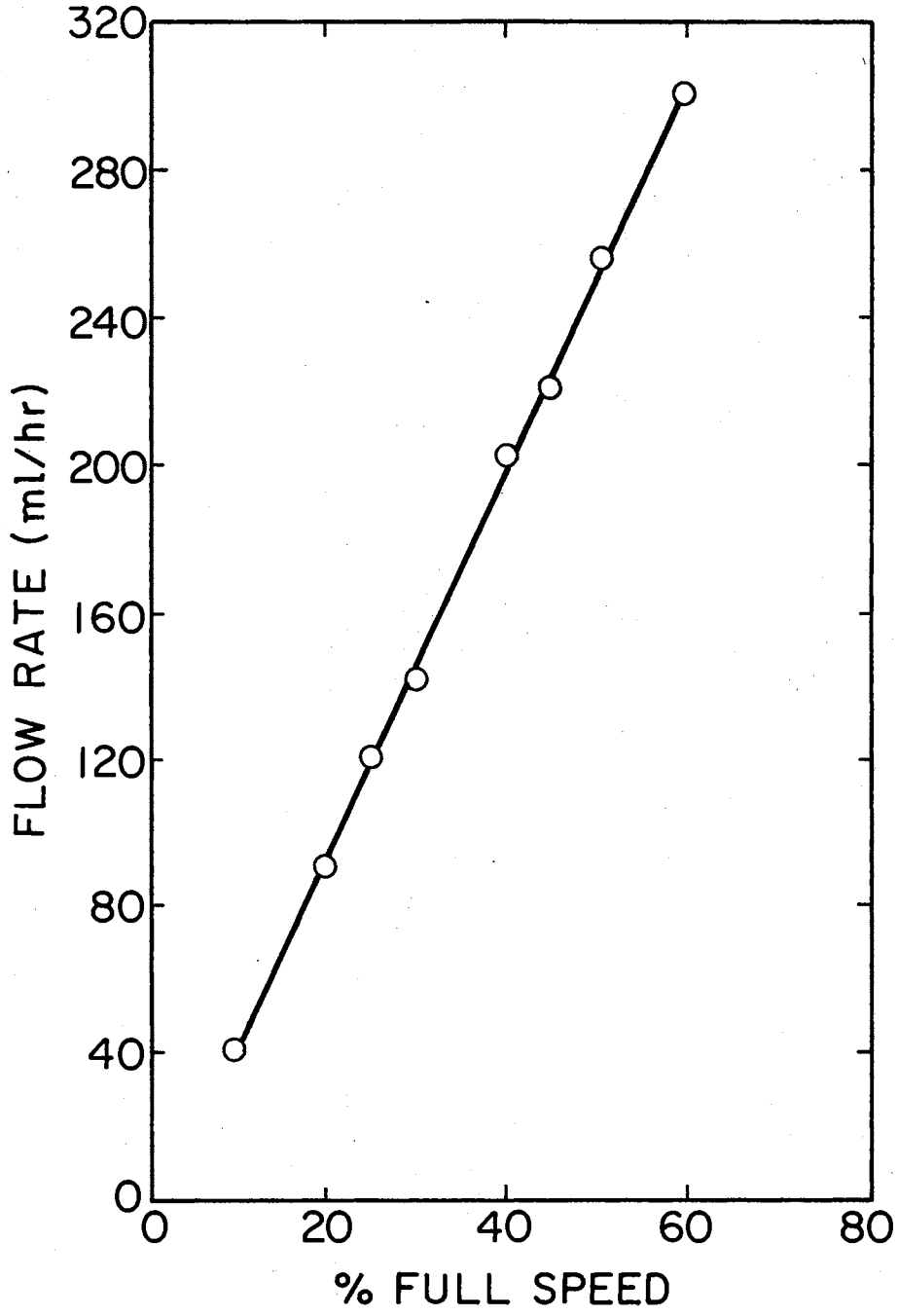

Figure 1. Calibration of the Sigmamotor Pump

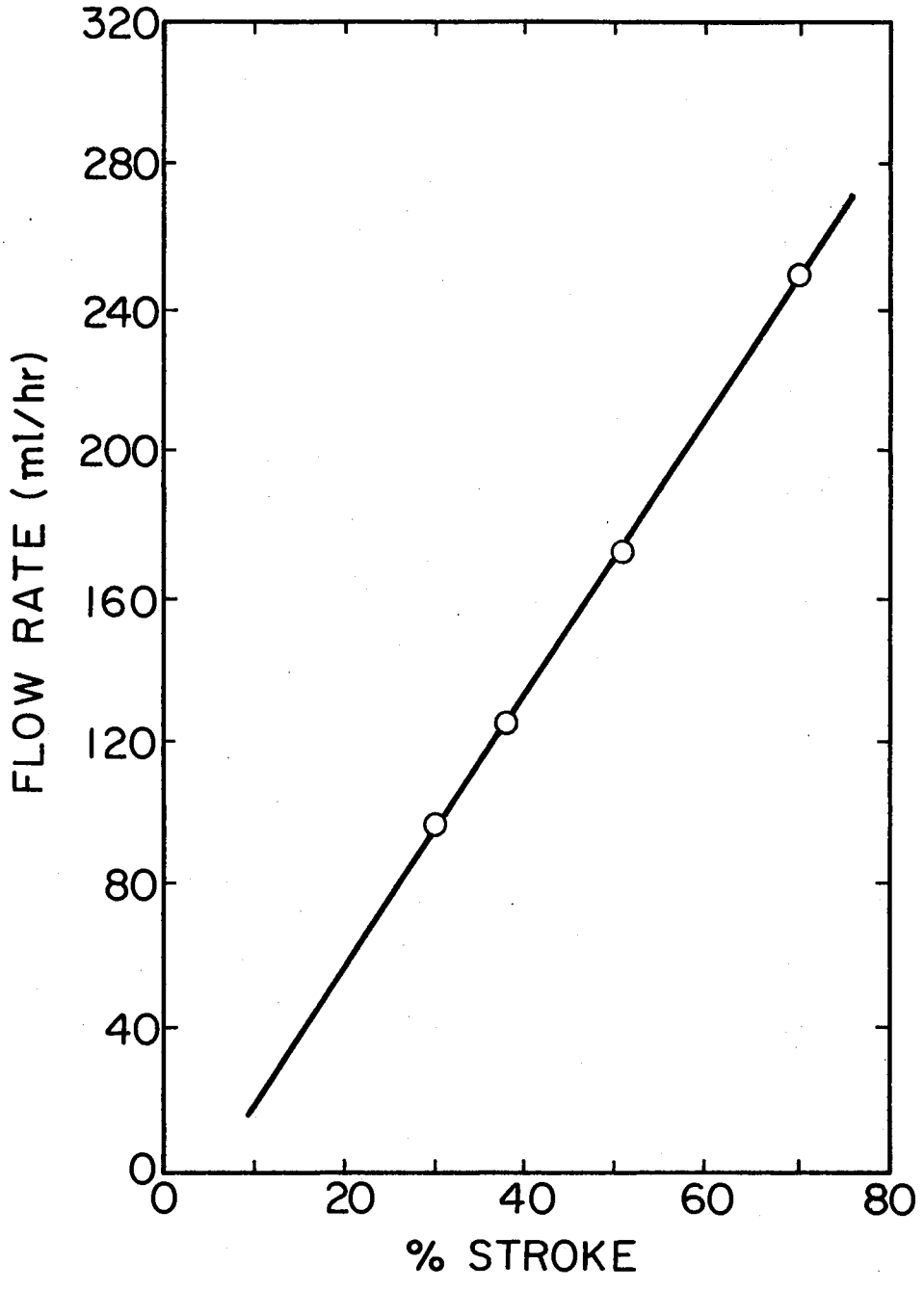

Figure 2. Calibration of the Milroyal Pump

TABLE II

DILUTION RATE OF SIGMAMOTOR PUMP

Pumping Rate % of Capacity	Flow Rate (ml/hour)	Dilution Rate (per hour)
10	38	.038
20	90	.090
30	145	.145
40	200	.200
50	255	.255

TABLE III

DILUTION RATE OF MILROYAL PUMP

Pumping Rate % of Capacity	Flow Rate (ml/hour)	Dilution Rate (per hour)
15	40	.040
20	60	.060
30	98	.098
50	172	.172
70	250	.250
90	320	.320

Foaming of the culture presented a problem until correction was made by using Dow Corning silicone antifoam. One attempt at direct use of antifoam into the culture resulted in contamination. Subsequent experiments involved spraying the antifoam into the pre-sterilized chemostat. This eliminated the problem although slight foaming did occur after approximately a week. However, this presented no serious problem as most experiments were completed within this time.

For the type of continuous flow apparatus used in these studies, it was found that experiments were best completed within ten days. Data collected after this time would undoubtedly be complicated by culture foaming and growth in the feed lines. The latter presented problems in nearly every experiment extended beyond one week. This was especially so when the culture was grown at a dilution rate of $1/24\ hr^{-1}$. A few experiments were terminated before completion because of this problem. Effect upon the culture was closely monitored during this period of growth and any evidence of steady state change necessitated experiment termination. No solution was found for this problem. However, most collected data were obtained during a time when feedline growth was not evident. The most direct effect of feedline growth on the culture was alteration of dilution rate primarily as a result of blocking the inflow tube on the chemostat cap. However in one instance, contamination of the feed reservoir occurred. This resulted in termination of that particular experiment.

Optical density readings were performed on samples of the continuous culture from the time it was inoculated until conclusion of the experiment. Such readings presented a convenient method of following culture growth to a steady state from the time it was inoculated. It was also

useful as a means of evaluating the steadiness of the steady state as well as providing an indication of perturbances in the system. Indirect estimations of the dry weight and viable cell numbers could also be made from optical density readings. However, when either dry weight or a viable cell number was desired, direct measurements were performed rather than relying on the spectrophotometric technique. Dry weights measured directly were performed during the steady state to monitor the steadiness of the system.

Results of the continuous culture studies were compiled from data taken at dilution rates of either 1/6 hr^{-1} or 1/24 hr^{-1} as well as optical density transition readings. Steady state levels of cells at either dilution rate were achieved within 24 to 48 hours after inoculation of the chemostat. Steady state was assumed when percent transmittance as measured by the spectrophotometer varied by no more than 1 unit over 24 hours. After the culture remained in steady state for at least 24 hours, samples were taken at different time intervals over the succeeding days for analysis of the yield constants. Steady state values of dry weight and residual COD for both the 1/6 hr^{-1} and 1/24 hr^{-1} dilution rates are presented in Figures 3 and 4. Each point on the curve represents a single sample taken for yield analysis within the 24 hour time interval. The dry weight for the 1/6 hr^{-1} dilution rate ranged from approximately 390 mg/l to 430 mg/l. Values for the 1/24 hr^{-1} dilution rate varied from 320 to 350 mg/l. Both ranges of values were acceptable as steady state values. The residual COD in the 1/6 hr^{-1} dilution rate ranged from 40 to 75 mg/l of unused substrate. The values for the 1/24 hr^{-1} dilution rate ranged from 75 to 103 mg/l of unused substrate. During these periods of COD measurement, 5 ml samples were

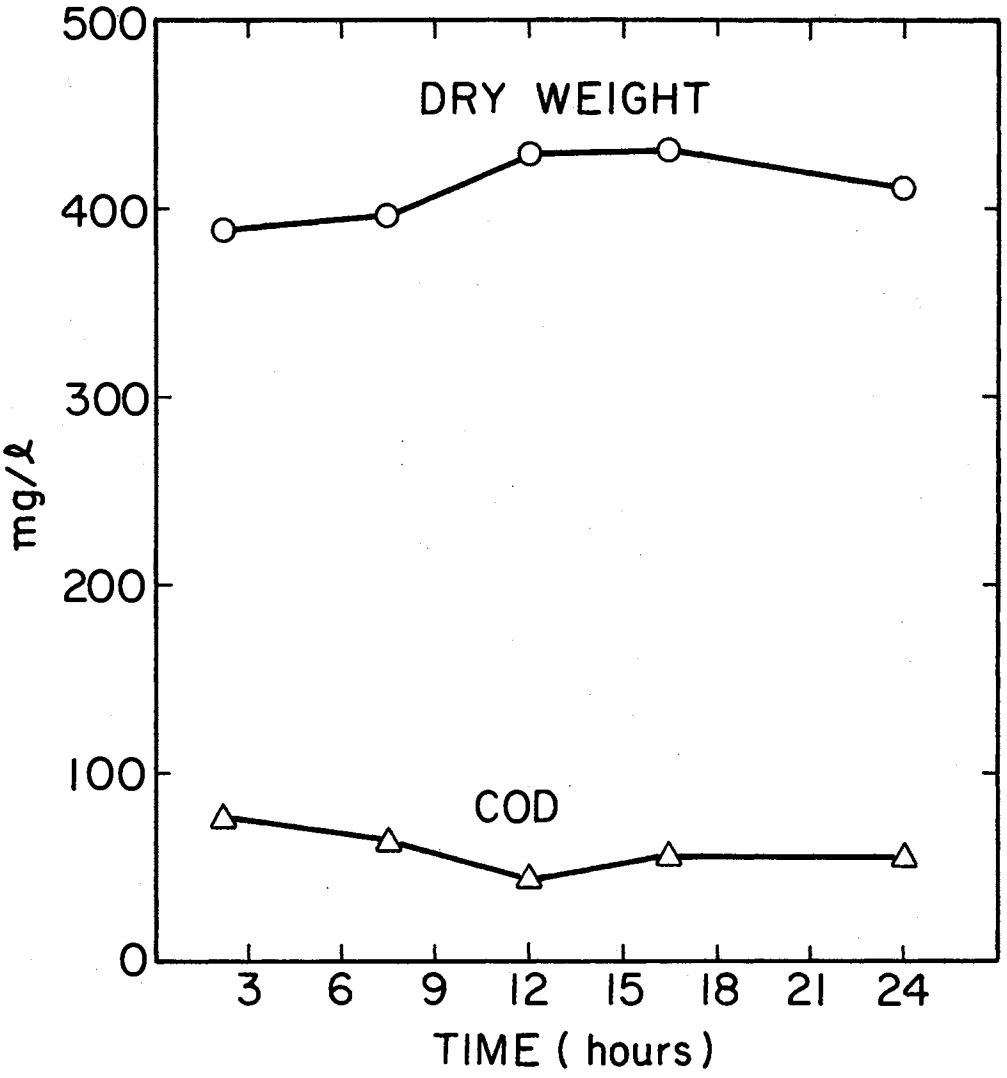

Figure 3. Steady State Values of Dry Weight and Residual COD in a Continuous Culture with a Dilution Rate of 1/6 hr^{-1} at 30°C

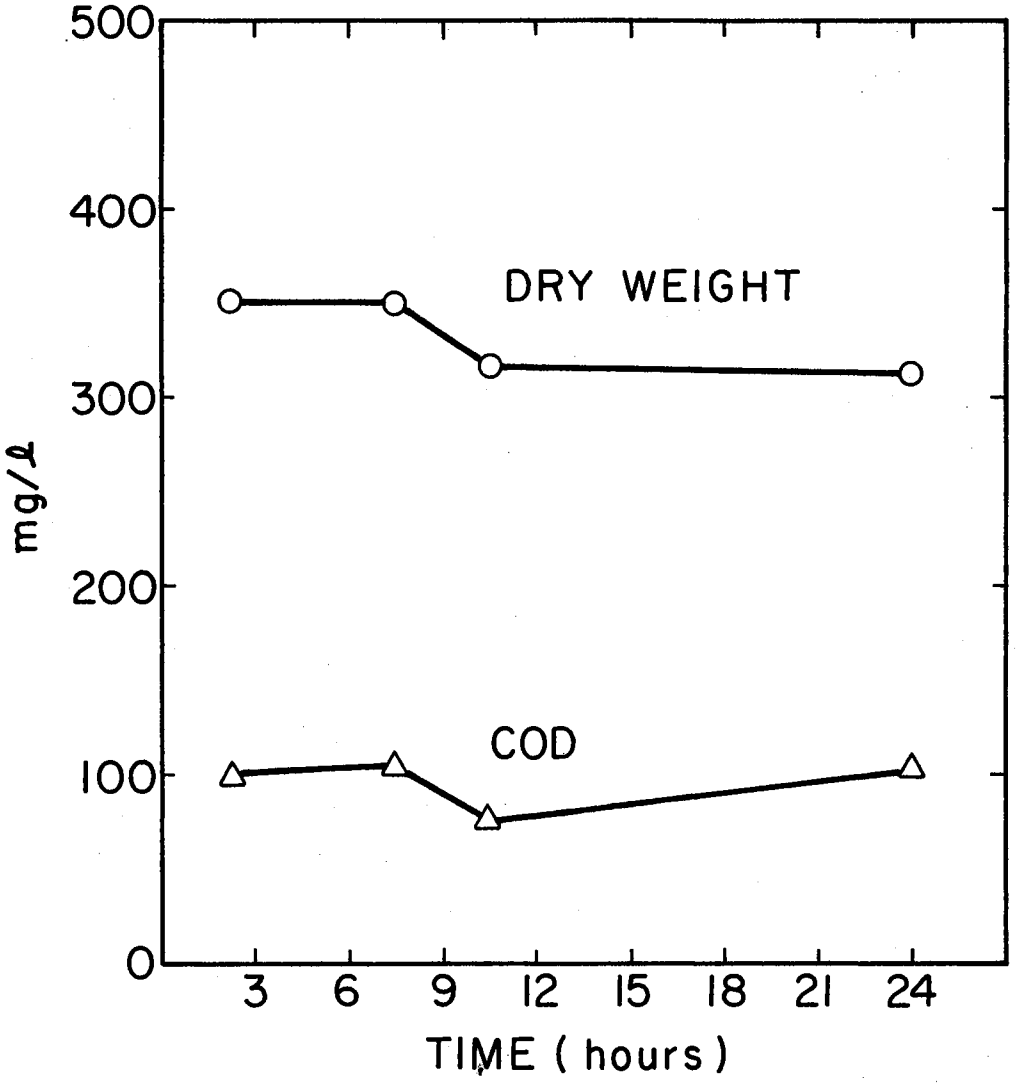

Figure 4. Steady State Values of Dry Weight and Residual COD in a Continuous Culture with a Dilution Rate of 1/24 hr^{-1} at 30°C

taken often as a continuing check on the steady state level. When experiments were completed at a particular dilution rate, the pump was readjusted so that the medium delivery resulted in a dilution rate of either 1/6 hr^{-1} or 1/24 hr^{-1} depending upon which was desired. The transient response of the culture going from one steady state level to another was followed by spectrophotometry on 5 ml samples collected during this time interval. These values are plotted in Figures 5 and 6 for the transient going from 1/6 hr^{-1} to 1/24 hr^{-1} dilution rate and from 1/24 hr^{-1} to 1/6 hr^{-1} dilution rate, respectively. The predicted response of each transient is confirmed upon the examination of both plots. The completion of the transient from the time the dilution rate was changed to the attainment of a new steady state level was about 27 hours for the transient of 1/6 hr^{-1} to 1/24 hr^{-1} and approximately 16 hours for the transient of 1/24 hr^{-1} to 1/6 hr^{-1}. The transition from 1/6 hr^{-1} to 1/24 hr^{-1} showed a gradual decrease in optical density until the new steady state level was attained. Incomplete mixing of the culture in one experiment resulted in an increase in optical density during the transient before the decrease but subsequent experiments showed the smooth gradual decrease as shown in Figure 5. Mixing was measured in the latter experiments by taking a sample from the overflow and comparing its optical density with a sample taken from near the bottom of the chemostat. This was done by using a syringe fitted to a piece of rubber tubing inserted through one of the inlet valves of the chemostat cap into the culture to a level just above the bottom. This procedure resulted in identical optical density

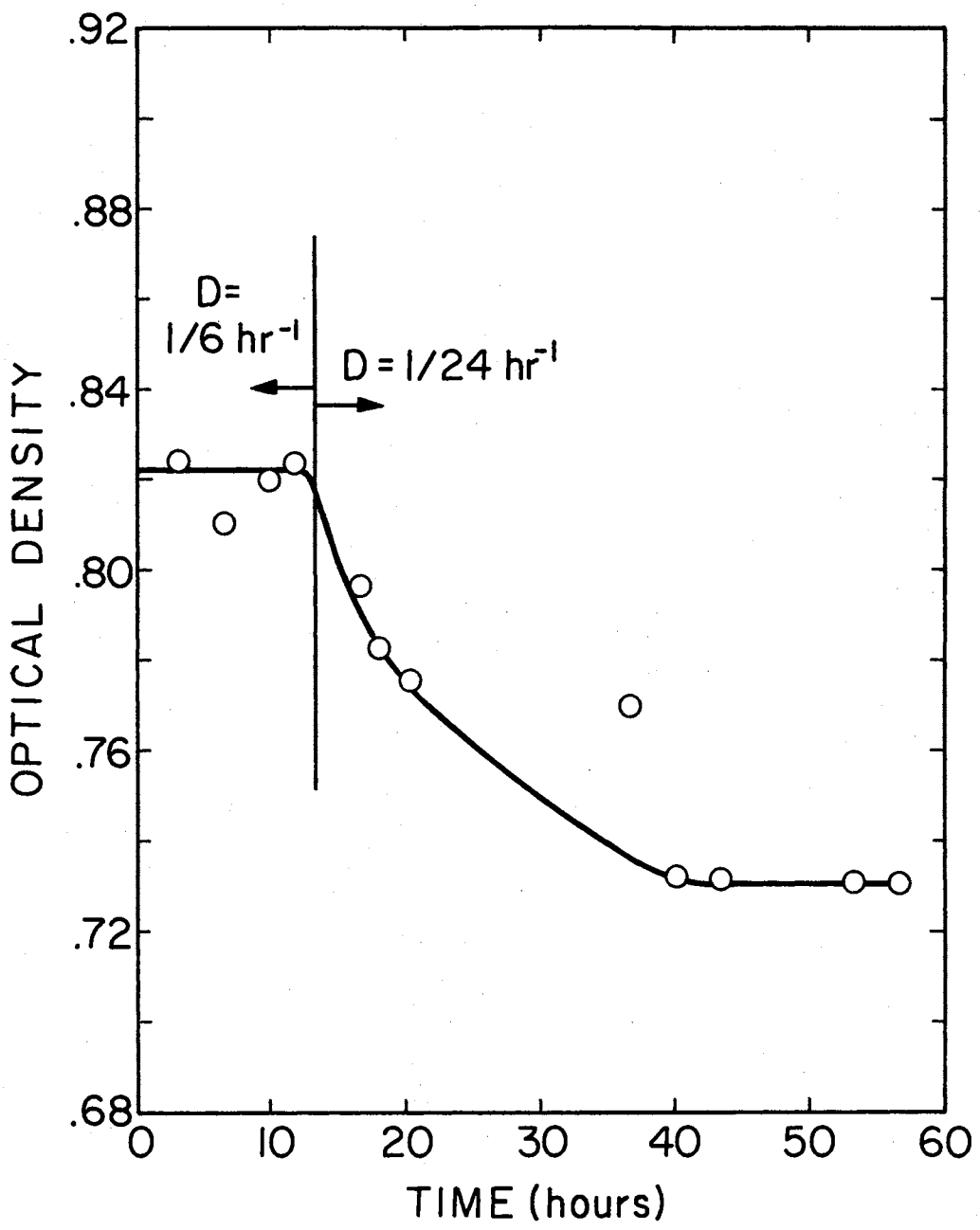

Figure 5. Optical Density Values for the Continuous Culture During the Transition from a Dilution Rate of 1/6 hr^{-1} to 1/24 hr^{-1} at 30°C

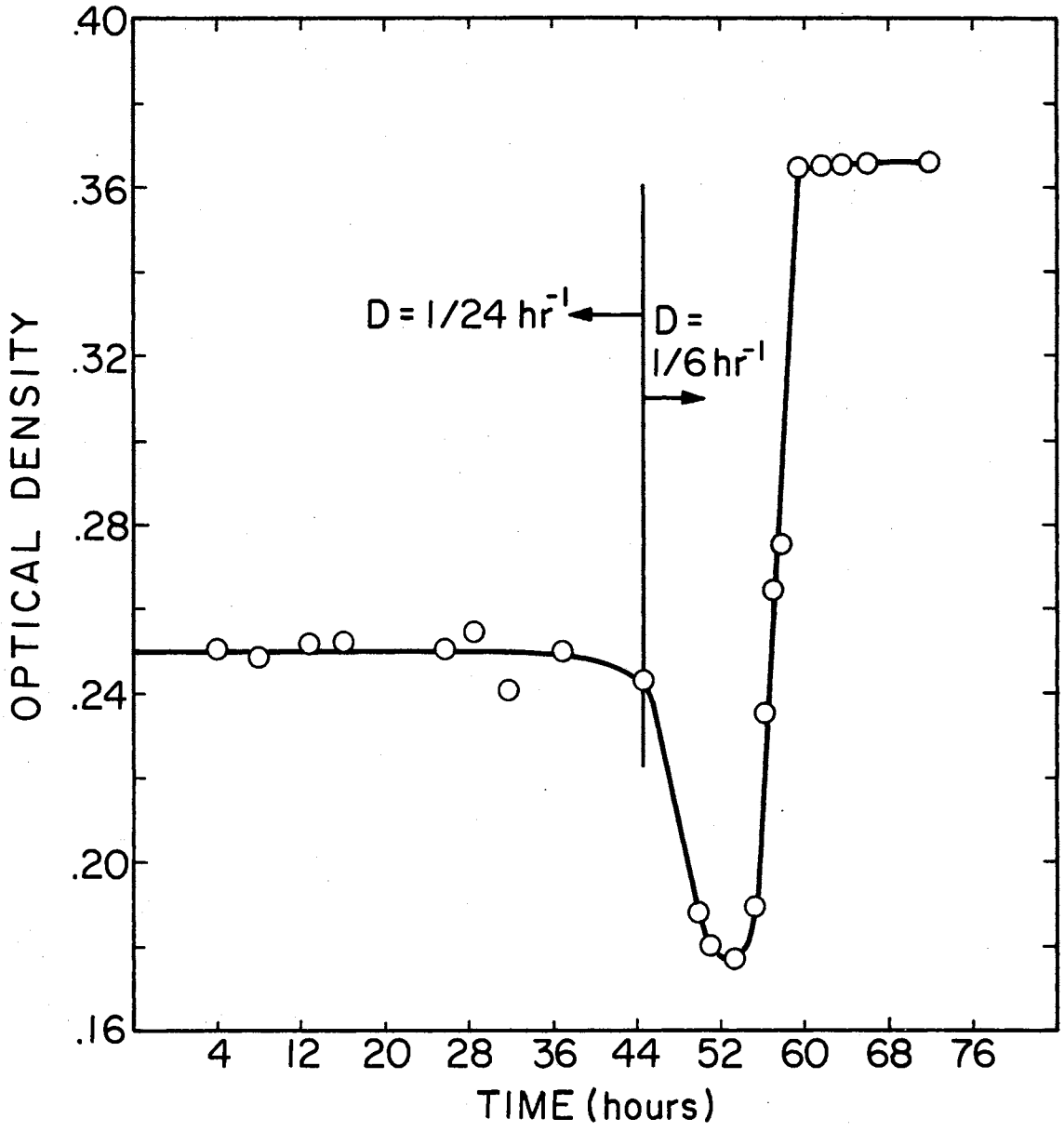

Figure 6. Transition Readings in the Continuous Culture Going from a Dilution Rate of 1/24 hr^{-1} to 1/6 hr^{-1} at 30°C

readings for both samples indicating adequate mixing was being attained in the chemostat. Several values were taken during the transient phase.

The plot for the transient from 1/24 hr^{-1} to 1/6 hr^{-1} in Figure 6 illustrates the effect of an increase in dilution rate upon a slowly growing culture. Upon changing the dilution to the faster rate there is an almost immediate wash-out of the cells which continues for about eight hours until the culture recovers and begins growing at a faster rate. The result is a new steady state at a cell density level that is higher than that at the dilution rate of 1/24 hr^{-1}. From the results of these two transients it is evident that upon changing the dilution rate a minimum of 24 hours should elapse before readings are taken for steady state analysis.

Yield analyses were performed on samples from the continuous culture grown at either 25 or 30°C and at dilution rates of 1/24 hr^{-1} and 1/6 hr^{-1}. Table IV presents a summary of the results from the continuous culture at both dilution rates as well as batch yield analyses for cultures inoculated from the indicated chemostat culture. The chemostat-grown cell yields, Y_C, did not seem to be affected by the temperature at which the cells were grown. At a dilution rate of 1/6 hr^{-1}, the chemostat yields averaged 0.38 mg/mg for both 30° and 25°C. The chemostat yields for the 1/24 hr^{-1} dilution rate averaged 0.32 mg/mg for both temperatures. These differences in yield were significant because no overlapping values were obtained in any of the determinations for the two dilution rates. Although the yields remained the same whether the temperature was 25° or 30°C, the residual COD at 25°C was very high compared to that at 30°C. Apparently, the lower temperature affected the physiology of the cells, resulting in less utilization of

TABLE IV

EFFECT OF SPECIFIC GROWTH RATE, μ, ON CELL YIELD DURING CONTINUOUS GROWTH AND BATCH GROWTH

	$D = 1/6\ hr^{-1}$ (0.17)			$D = 1/24\ hr^{-1}$ (0.041)		
Temperature	Y_C[1] mg/mg	Y_B[2] mg/mg	μ hr^{-1}	Y_C mg/mg	Y_B mg/mg	μ hr^{-1}
30°	0.38	0.38	0.57	0.32	0.33	0.52
25°	0.38	0.37	0.28	0.32	0.32	0.28

Abbreviations: Y_C, continuous flow cell yields; Y_B, batch yields; μ, specific growth rate, batch culture.

[1]All continuous flow yield values were an average of 4 - 12 separate determinations.

[2]All batch yield values were an average of 5 - 12 separate determinations.

the available substrate. At the conclusion of the 25°C series of experiments, subsequent studies were performed at 30°C where the residual substrate was quite low at both dilution rates. The steady state levels at this temperature also appeared to be more stable when monitored by spectrophotometry. The batch flasks inoculated from the continuous culture and grown at 25°C also left much unused substrate after their log phase was completed. The remainder of the data presented was collected at 30°C.

The batch yields, Y_B, for the corresponding chemostat dilution rates present an interesting observation. Y_B equals Y_C for a given chemostat dilution rate. These results were unexpected with a pure culture because specific growth rate in the batch experiments was considerably higher than those in the chemostat at either dilution rate. For the 1/6 hr^{-1} chemostat dilution rate the corresponding specific growth rate in batch was 0.57 hr^{-1} or about three times that in the chemostat. For the 1/24 hr^{-1} chemostat dilution rate the corresponding batch specific growth rate was over ten times that in the chemostat. Maintenance theory predicts that the higher the specific growth rate the higher the yield because a minimum portion of the energy source is used for maintenance of the cell at high specific growth rates while an increasing proportion is used for maintenance as the specific growth rate becomes slower. The results summarized in Table IV do not agree with this concept when Y_C is compared to Y_B. However, the comparison of Y_C's from different dilution rates illustrate the effect predicted by the maintenance theory. The same effect was noted at both 30°C and 25°C for a number of experiments. The major portion of the ensuing experiments were performed in an attempt to explain the results reported herein.

As a means of varying specific growth rate so that yields at these growth rates could be determined, a series of batch flasks were set up with varying concentrations of the limiting carbon and energy source, glucose. The concentrations of glucose ranged from 50 to 1000 mg/l. However, the concentrations below 300 mg/l presented difficulty in measurement of specific growth rate because of their extremely short growth phase which was difficult to follow by optical density. Specific growth rate was determined as outlined in the methods section. The effect of specific growth rate on yield is presented in Table V. Replicates of each experiment were performed and presented as experiments I and II in the table for each chemostat dilution rate. Two important findings resulted from this experiment. First, it was shown that specific growth rate depended upon and could be controlled by initial substrate concentration. Measurement of the specific growth rate showed a definite log phase for each substrate concentration even though specific growth rate was below μ_{max}. Second, the substrate-based cell yield remained constant for each specific growth rate and substrate concentration. More specifically, the yield retained the same value as the yield in the chemostat from which the flasks were inoculated. It is apparent from these results that the specific growth rate had no effect upon the yield as would be predicted by the maintenance theory. This observation was experimentally repeatable.

The effect of initial substrate concentration on the rate and total amount of microbial growth in batch with inocula from the chemostat is shown in Figures 7 and 8 as units of optical density. Each substrate concentration represented on the two plots was characterized by a log phase of a definite duration. Total growth measured directly as

TABLE V

EFFECT OF SPECIFIC GROWTH RATE ON YIELD
IN BATCH CULTURES AT 30°C

6 Hour Batch[1] - Experiment I			6 Hour Batch - Experiment II		
S_i (mg/l)	μ (hr^{-1})	Y (mg/mg)	S_i (mg/l)	μ (hr^{-1})	Y (mg/mg)
1000	0.60	0.37	1000	0.53	0.38
800	0.57	0.36	900	0.50	0.37
700	0.55	0.37	800	0.50	0.36
600	0.53	0.35	700	0.46	0.35
500	0.50	0.38	500	0.42	0.36
300	0.43	0.40	400	--	0.38
24 Hour Batch[2] - Experiment I			24 Hour Batch - Experiment II		
1000	0.48	0.33	900	0.55	0.32
900	0.46	0.32	800	0.55	0.32
800	0.46	0.34	700	0.55	0.31
700	0.43	0.32	500	0.53	0.31
500	0.42	0.32	400	0.43	0.32
300	0.43	0.33	300	0.39	0.27

[1]6 hour batch - inoculum for flasks was taken from chemostat operated with 6 hour detention time, $D = 1/6\ hr^{-1}$.

[2]24 hour batch - inoculum for flasks was taken from chemostat operated with 24 hour detention time, $D = 1/24\ hr^{-1}$.

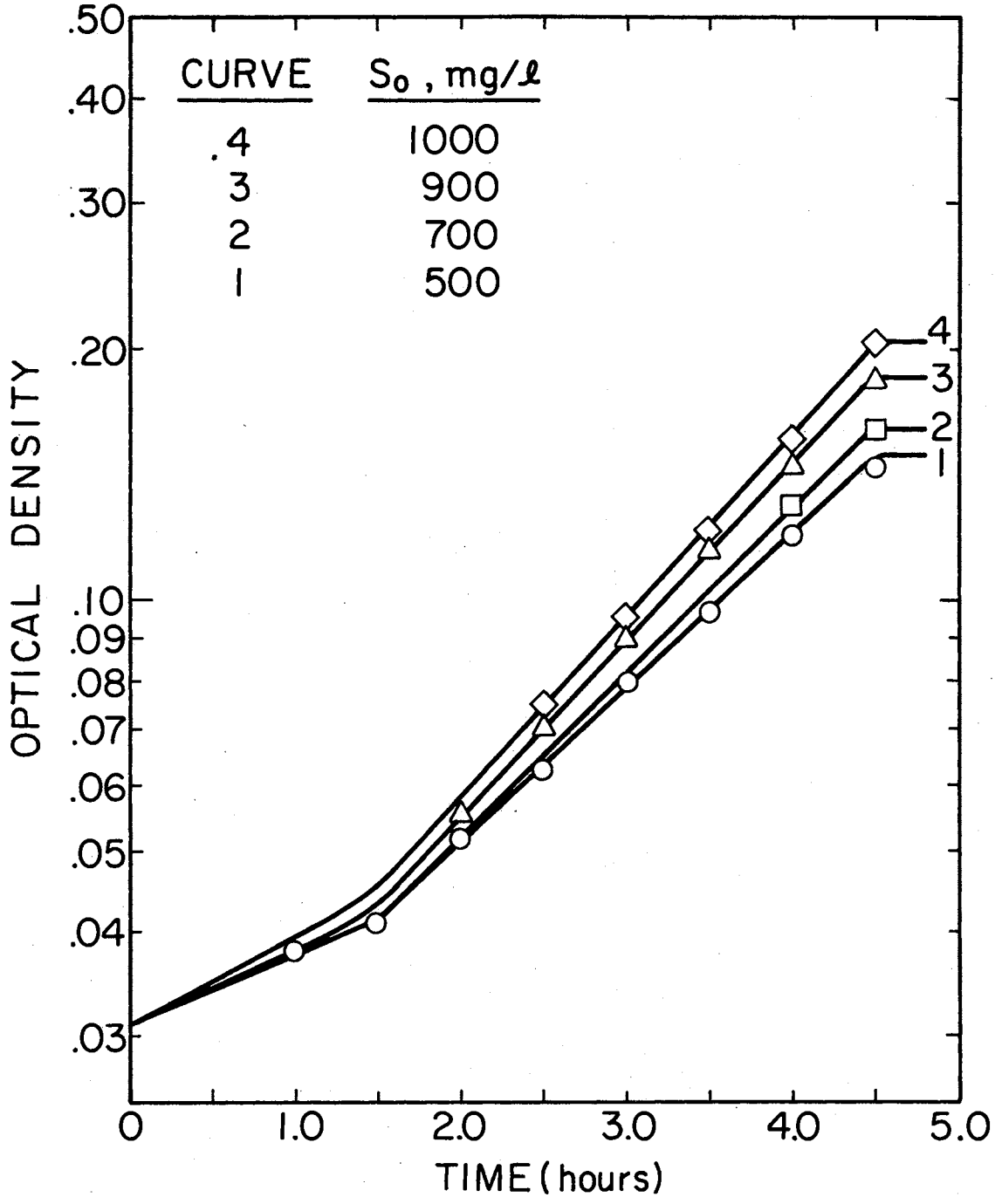

Figure 7. Effect of Initial Substrate Concentration S_0 on the Rate and Total Amount of Microbial Growth in Batch with Inocula from a Continuous Culture at a Dilution Rate of 1/6 hr^{-1} at 30°C

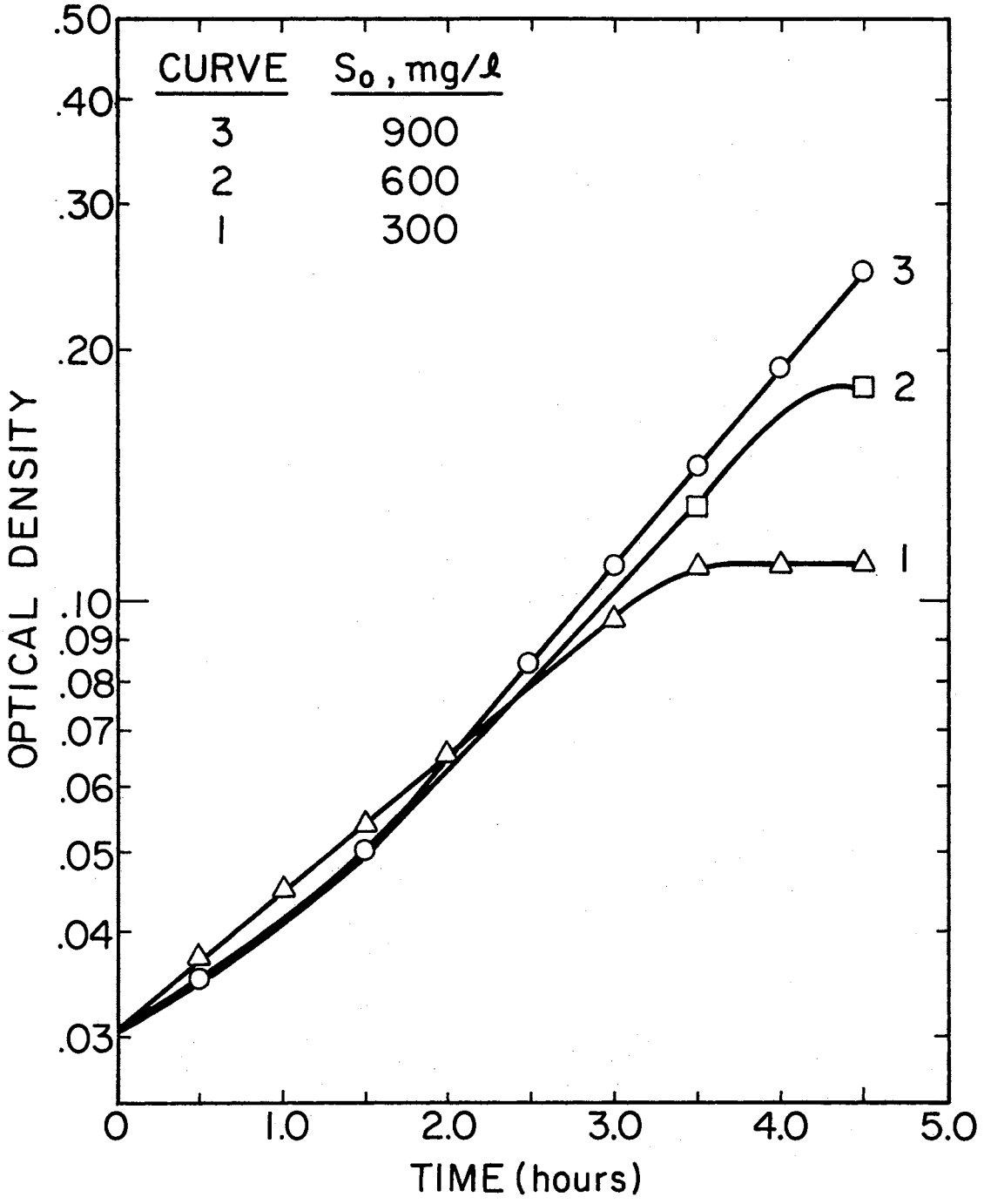

Figure 8. Effect of Initial Substrate Concentration S_0 on the Rate and Total Amount of Microbial Growth in Batch with Inocula from a Continuous Culture at a Dilution Rate of 1/24 hr^{-1} at 30°C

optical density increased with increasing substrate concentration. The final growth is represented on the two plots by the plateau which followed the log and deceleration phases. It was determined experimentally although for space considerations the points defining it are not shown. Calculations of specific growth rates were made from graphs of this type for each substrate concentration.

The growth constants, K_S and μ_{max}, were calculated from data collected from the replicate experiments as summarized in Table V. A regression line was calculated for each dilution rate by combining the results of both experiments and determining the line by the method of least squares. Figures 9 and 10 represent double reciprocal plots of the specific growth rate versus substrate concentration for the 1/6 hr^{-1} and 1/24 hr^{-1} dilution rates respectively. The calculated regression line is drawn on the plots with the experimental points of two separate experiments entered on each. The reciprocal of the intercept is defined as the μ_{max} and was determined for both chemostat dilution rates. The K_S was determined from both the slope and μ_{max} by multiplying the two. For the batch flasks inoculated from the chemostat with a dilution rate of 1/6 hr^{-1}, the μ_{max} was 0.64 hr^{-1} and the K_S was 171 mg/l. The growth studies inoculated from the 1/24 hr^{-1} chemostat resulted in a calculated μ_{max} of 0.58 hr^{-1} and a K_S of 133.4 mg/l. Theoretically, the growth constants should be identical for cells grown at both specific growth rates but the results from this experiment showed there is a difference depending upon previous specific growth rate. Each pair of growth constants was utilized for calculating the curves shown in Figures 11 and 12. The constants were substituted into the Monod equation and the calculated curve was drawn as shown for both experiments. The actual

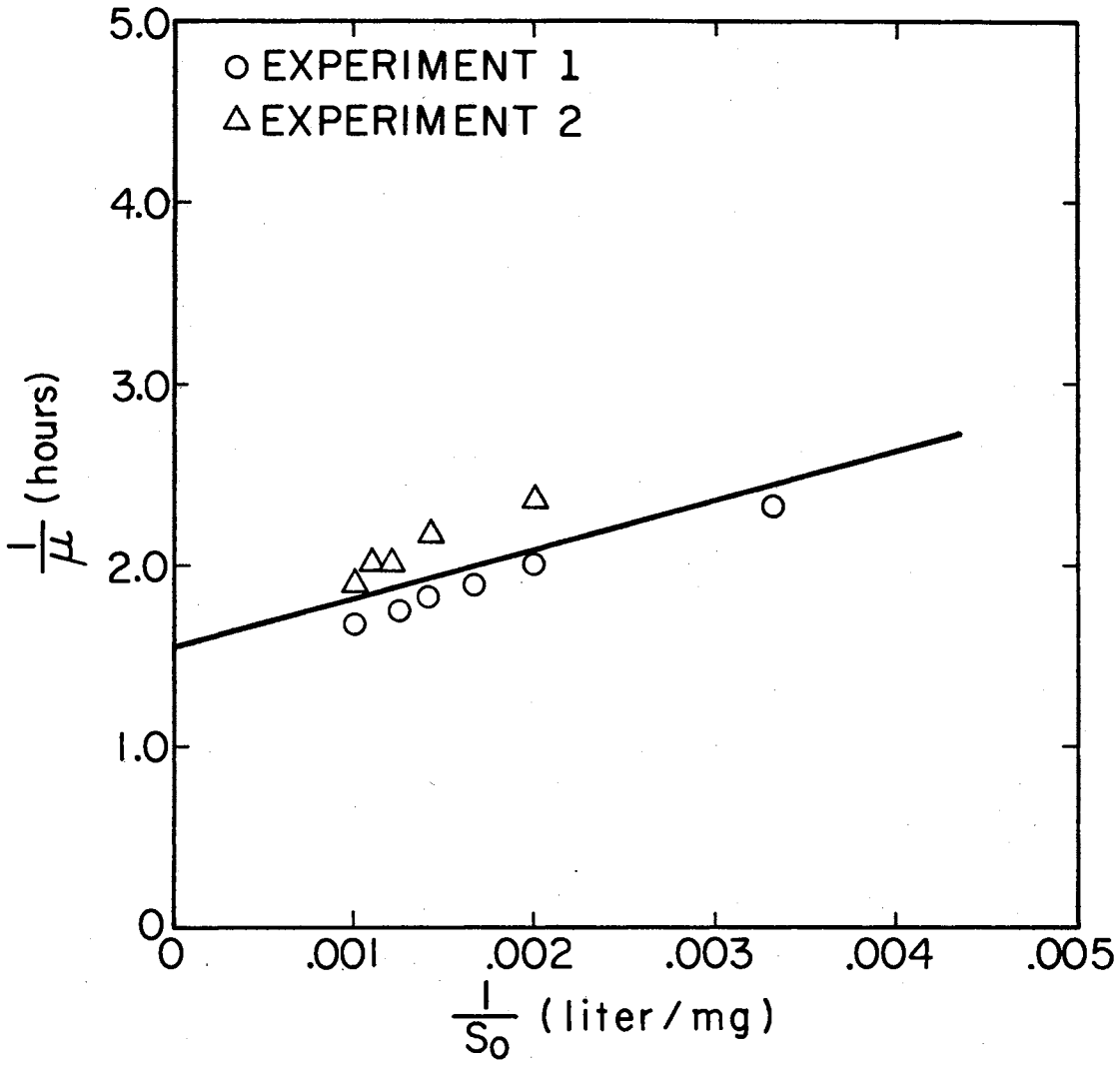

Figure 9. Variation of Specific Growth Rate, μ, with Initial Substrate Concentration, S_0, Plotted as Double Reciprocals for Estimation of K_S and μ_{max} in Batch Experiments Inoculated from a Continuous Culture at a 1/6 hr^{-1} Dilution Rate at 30°C

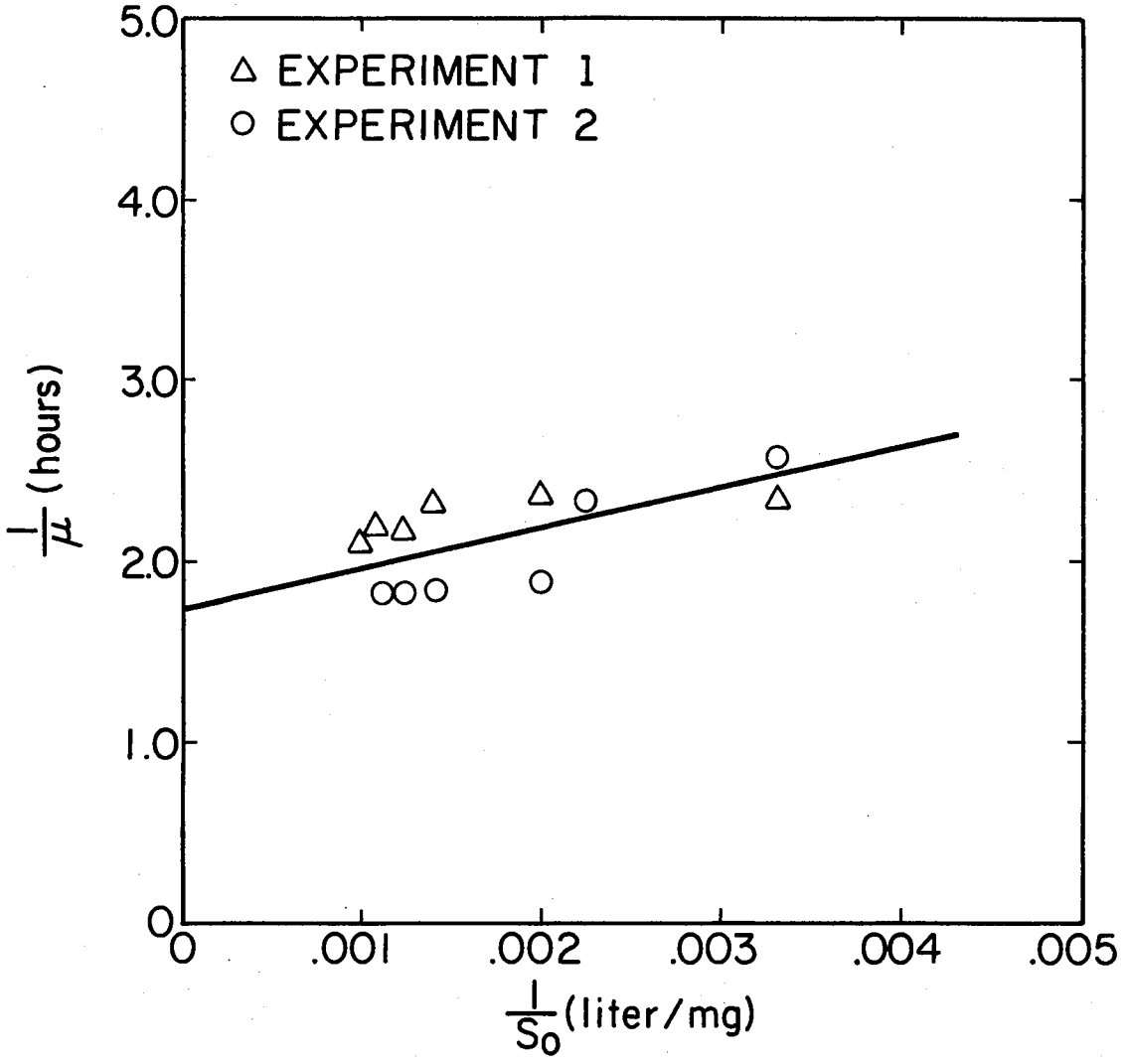

Figure 10. Variation of Specific Growth Rate, μ, with Initial Substrate Concentration, S_0, Plotted as Double Reciprocals for Estimation of K_S and μ_{max} in Batch Experiments Inoculated from a Continuous Culture at a 1/24 hr^{-1} Dilution Rate at 30°C

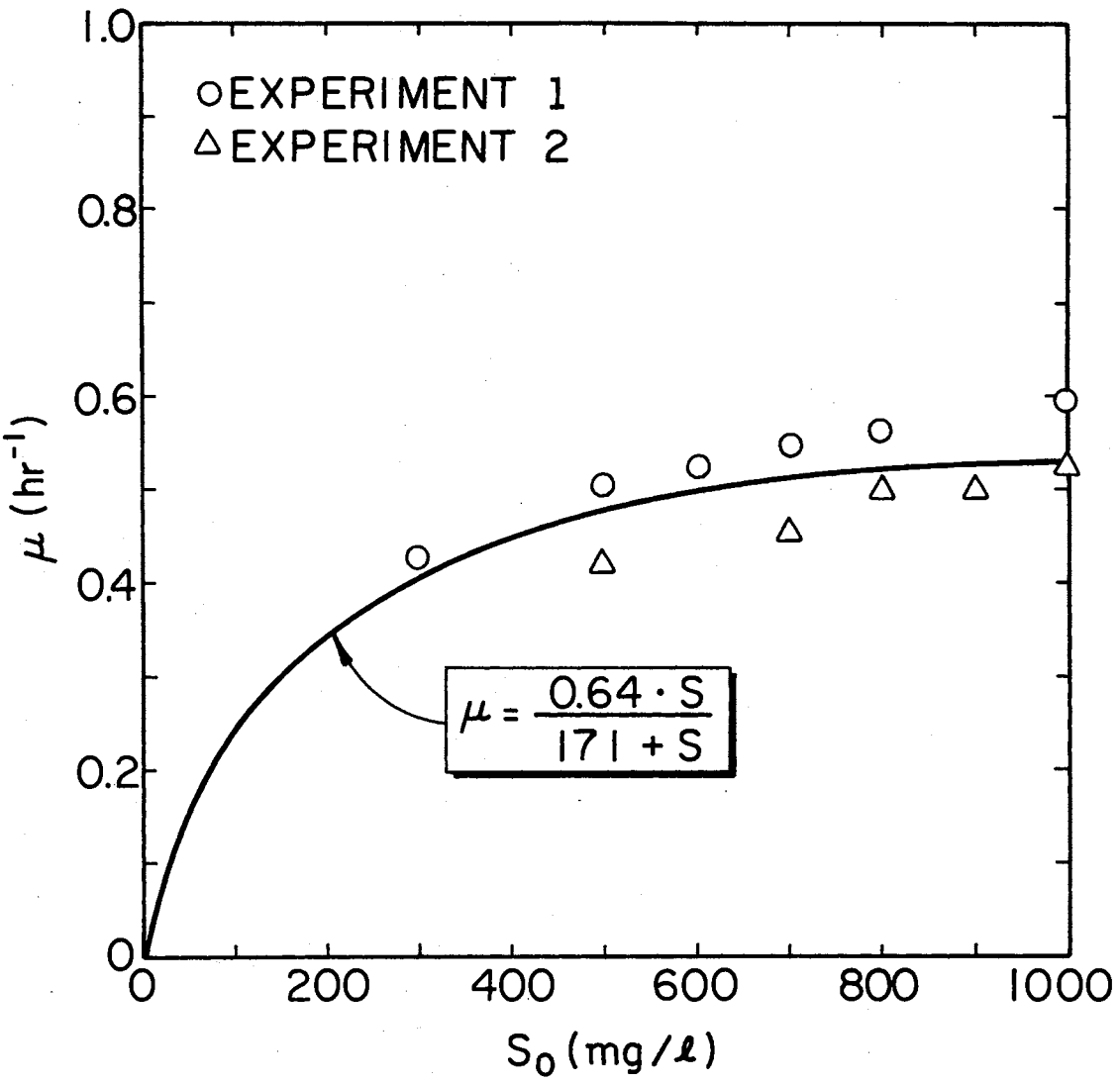

Figure 11. Variation of Specific Growth Rate, μ, with Initial Substrate Concentration, S_0, for Batch Experiments Inoculated from a Continuous Culture with a Dilution Rate of 1/6 hr^{-1} at 30°C

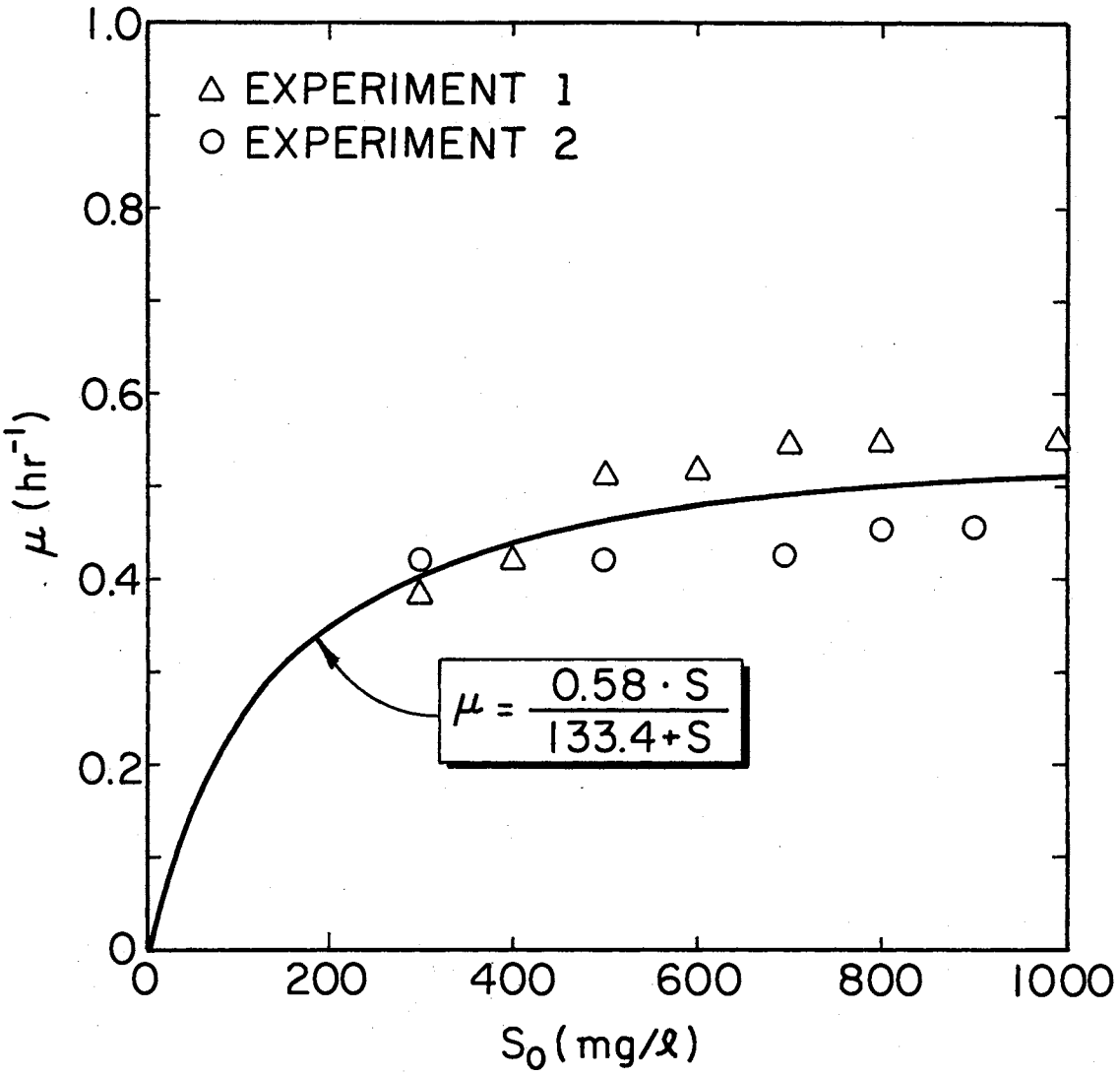

Figure 12. Variation of Specific Growth Rate, μ, with Initial Substrate Concentration, S_0, for Batch Experiments Inoculated from a Continuous Culture with a Dilution Rate of 1/24 hr^{-1} at 30°C

experimental results of two separate determinations are also represented on the plot for each dilution rate. Both curves show the variation of specific growth rate with initial substrate concentration. At the higher concentrations of substrate the specific growth rate becomes independent of the substrate concentration and approaches μ_{max}.

Experiments were designed to determine if the batch yield remained the same through two log phases. Batch flasks were inoculated from the chemostat and the specific growth rate and yield were determined for each flask. Near the end of log phase a new series of batch flasks was inoculated from these flasks and grown through another log phase. The results of these experiments are shown in Table VI as replicates of each study for the two dilution rates. There seems to be no effect of repeated batch transfer on cell yields at least through two log phases. Both transfers for each experiment resulted in approximately the same yields with no tendency for the yield to return to the higher yields expected when growth is at or near μ_{max}. Measurement of μ in each experiment showed that high specific growth rates were present in each experiment. The yields in this segment of the studies were higher than in earlier studies reported herein. However, the effect of chemostat dilution rate on yields in both the chemostat and batch remained the same.

At the time these studies were done, plating on EMB lactose plates revealed in addition to the normal colonies some colonial morphological variants which were present in a ratio of about 10 normal colonies to 1 variant. The normal colonies are herein referred to as R and the variants are referred to as the S colonial strain. The same tests used for the identification of the isolated E. intermedia strain were

TABLE VI

EFFECT OF REPEATED BATCH TRANSFER ON CELL YIELDS FROM THE CONTINUOUS CULTURE

Y_C (mg/mg)	Transfer	Y_{B_1} (mg/mg)	μ (hr^{-1})	Y_{B_2} (mg/mg)	μ (hr^{-1})
0.44[1]	1	0.44	0.54	0.42	0.51
--	2	0.40	--	0.41	0.59
0.55[2]	1	0.54	0.61	0.53	0.57
--	2	0.55	0.70	--	--

Abbreviations: Y_C, continuous flow cell yield; Y_{B_1}, batch yield, replica I; Y_{B_2}, batch yield, replica II.

[1]Yield analyzed from a continuous culture grown at a dilution rate of 1/24 hr^{-1}.

[2]Yield analyzed from a continuous culture grown at a dilution rate of 1/6 hr^{-1}.

performed on the S colony type. The results showed it to have identical characteristics to the R strain. The S strain made mucoid colonies and initially remained stable as an S type. However, reculturing unfortunately resulted in its reversion to the common R type before completion of all studies involving it.

Experiments were designed involving the two culture types to ascertain the effect of the S type on the yield. The appearance of the S type colonies occurred before the time of the higher yield observation. All culture conditions remained the same throughout all experiments. Evidently, a change in the test organism resulted in the higher yield. Batch flasks were prepared each containing 1000 mg/l of glucose. Inocula were prepared by separately growing the R and S cultures overnight in the minimal medium. A series of flasks for each culture type were inoculated at the same time and placed on the shaker at 30°C. Specific growth rates were determined from the optical density versus time plots. The results of these experiments are shown in Table VII. It is evident the specific growth rates were nearly identical for the R and S culture types in any particular experiment. Each experiment listed is the average of triplicate determinations for both the yield and specific growth rate. A comparison of the average yields for the R and S culture types can be made from the table. Although the comparative yields are very similar, a trend may be noted. The yields for the R culture were, in every experiment, slightly higher. The statistical significance of these data was not determined.

Further experiments were performed with the R and S types to determine the effect of continuous culture on a mixed population of the two. No further yield or batch studies were done at this time. The main

TABLE VII

BATCH YIELD COMPARISON BETWEEN R AND S

Experiment[1]	Y_S (mg/mg)	μ (hr^{-1})	Y_R (mg/mg)	μ (hr^{-1})
1	0.43	0.54	0.44	0.54
2	0.42	0.55	0.44	0.54
3	--	0.69	0.42	0.69
4	0.40	0.69	0.43	0.69
5	0.40	0.63	0.42	0.63
6	0.41	0.63	0.45	0.46

Abbreviations: Y_S, batch yield of strain S; Y_R, batch yield of strain R.

[1]Each experiment involved triplicate determinations of yield in minimal medium with 1000 mg/l of glucose.

point of interest was the effect of dilution rate upon the predominance of one or the other culture type. Approximately equal inoculations were made from each culture into the chemostat after overnight growth on the shaker in minimal medium. Optical density was the criterion for determining the equal concentrations. Figure 13 shows the effect of dilution rate on the mixed culture. Plate counts were made on TSA plates in triplicate and incubated at 37°C for 18 hours. The two colony types could be distinguished on these plates within this time period. EMB lactose plates were also used but they resulted in consistently lower plate counts than did TSA plates so results were based upon the latter counts. The plate counts of the R variant remained fairly constant at both dilution rates although there was a slight increase in numbers at the 1/24 hr^{-1} dilution rate. The S variant decreased in numbers almost immediately at the 1/6 hr^{-1} dilution rate. It continued this decrease for three days until the dilution rate was changed to 1/24 hr^{-1}. A gradual increase in numbers occurred over the following six days until the system was shut down because of clogged feed lines. The numbers of S never reached the inoculum level although they were increasing in plate count at the time of termination. Table VIII presents a summary of the average plate counts for both dilution rates. The total plate count is also given. Interestingly, the plate counts at the 1/24 hr^{-1} dilution rate were higher than the total number at the 1/6 hr^{-1} dilution rate.

Another series of experiments was undertaken to ascertain a difference between the R and S culture types. Since all the tests used for identification of the original organism resulted in identical reactions, phage experiments were introduced to determine if phage

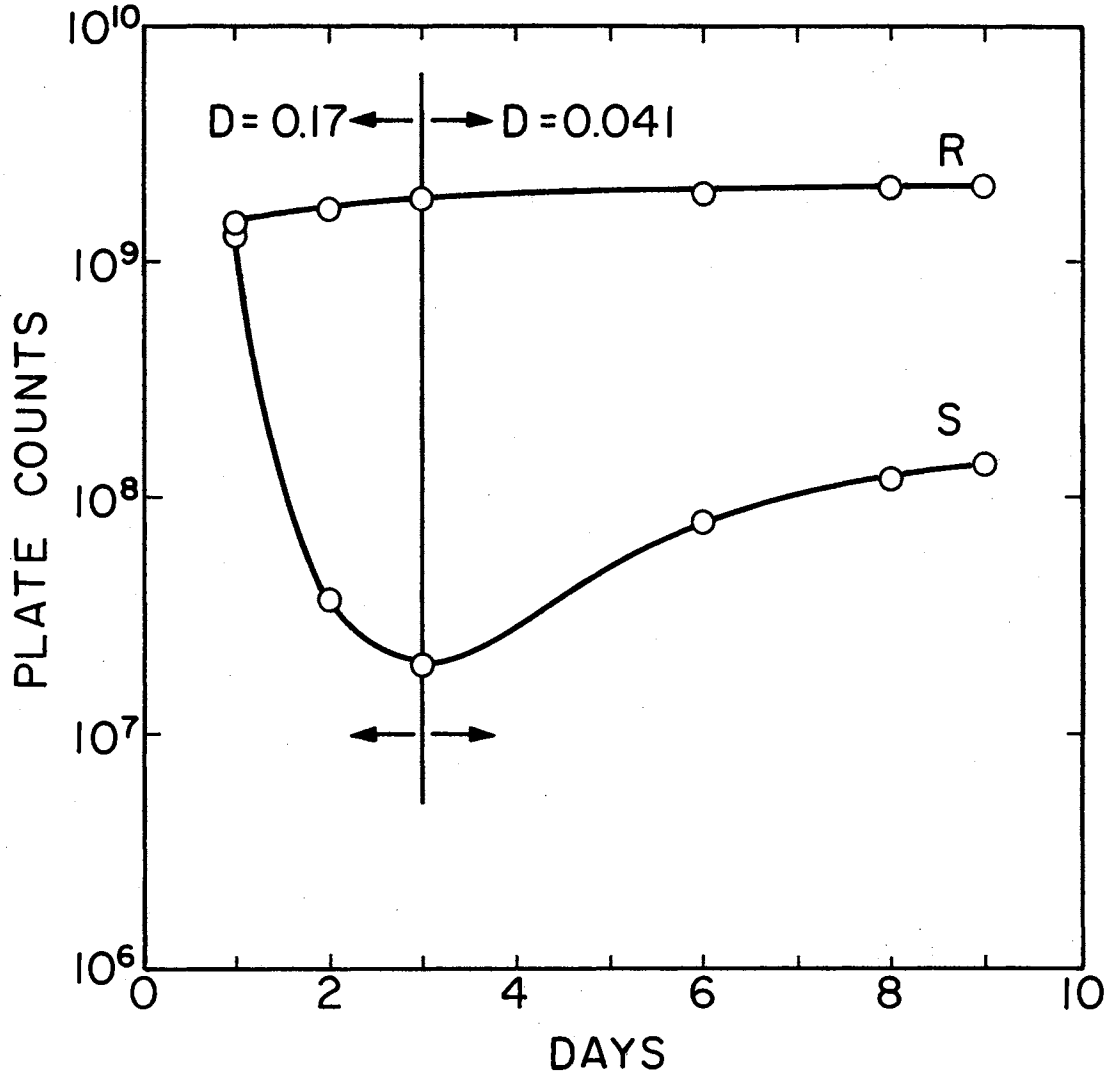

Figure 13. The Effect of Dilution Rate on a Continuous Culture with a Mixed Culture of R and S Colony Types

TABLE VIII

THE EFFECT OF DILUTION RATE ON THE RATIO OF R AND S COLONY TYPES GROWN IN A CONTINUOUS CULTURE

Colony Type	D = 0.17 Day 1	D = 0.17 Day 2	D = 0.17 Day 3	D = .041 Day 6	D = .041 Day 8	D = .041 Day 9
S	1.09×10^9	5.6×10^7	3.3×10^7	9.0×10^7	1.2×10^8	1.4×10^8
R	1.36×10^9	2.05×10^9	2.56×10^9	2.8×10^9	3.2×10^9	3.18×10^9
Total	2.45×10^9	2.1×10^9	2.59×10^9	2.89×10^9	3.32×10^9	3.32×10^9

The days are numbered from the time at which the continuous culture was set at a dilution rate of 0.17 through the period at which the dilution rate was changed to 0.041 on the third day. Each value for cell count was the average of three determinations.

attachment sites were dissimilar in the two culture types. Standard phage techniques were employed as described in the methods section herein. Eight bacteriophages were isolated for testing from municipal sewage. The plaque morphology as well as the bacterial culture type on which it was isolated are given in Table IX. A number of plaques were obtained on both culture types but only those with obvious differences in morphology were selected. They ranged in size from tiny to large and in turbidity from clear to very turbid. The phages were then purified and titers were determined for each plate stock. These are presented in Table X. These titers were used for determining the dilutions required for plating on the bacteria. Phage sensitivity of R and S were then determined by plating each phage in triplicate on each culture type. The growth of R-isolated phages on the R plates was used as a control as well as the S-isolated phages on the S plates. The purpose was to determine if both culture types had equal sensitivities to all phages. The results of these studies are shown in Table XI. Six of the eight isolated phages proved specific for a particular culture type and would not grow on the other. Only isolated phages S_8C and S_9C were able to grow on both R and S. Further experiments involving R and S were prevented because of reversion of the S type to the R type at such a rate that separation was impossible.

A final phase of this study was undertaken to determine the effect of washing upon the final yield. Also a special medium was prepared containing extra magnesium and ammonium sulfate to assure that neither were limiting growth in any way. Yields and specific growth rates were determined in the usual manner. Washing of the biological solids on the filter was either by distilled water or by 0.03 M sodium phosphate buffer

TABLE IX

PLAQUE MORPHOLOGY

Phage Isolate	Bacterial Colony Type	Morphology
R_1C	R	tiny, turbid
R_2C	R	tiny, clear
R_3C	R	tiny, clear center turbid edge
R_4C	R	very tiny, clear
S_5C	S	medium sized, slightly turbid
S_6C	S	large, clear center, turbid edge
S_8C	S	small, very turbid
S_9C	S	tiny, turbid

TABLE X

PHAGE PLATE STOCK TITERS

Phage	Titer
R_1C	5×10^9
R_2C	2×10^9
R_3C	8×10^8
R_4C	9×10^8
S_5C	1×10^9
S_6C	1×10^9
S_8C	4×10^9
S_9C	2×10^9

TABLE XI

R AND S PHAGE SENSITIVITY

Phage	Growth on S	Growth on R
R_1C	-	+
R_2C	-	+
R_3C	-	+
R_4C	-	+
S_5C	+	-
S_6C	+	-
S_8C	+	+
S_9C	+	+

at a pH of 7.1. Both minimal media were used for this test. The results are presented in Table XII. The flasks with the normal medium resulted in higher yields than did the enriched medium although the specific growth rates were approximately the same. These yields reflect the higher values of the constant which arose midway through these studies.

The washing solution appeared to have no consistent effect on the yield. Those cells grown in the normal medium had higher yields when buffer was used for washing but those grown in the magnesium and nitrogen enriched medium had equivalent yields for both washings.

TABLE XII

THE EFFECT OF WASHING SOLUTION AND EXTRA MAGNESIUM AND AMMONIUM SULFATE ON BATCH CELL YIELDS

Flask	Medium	$\mu(hr^{-1})$	Yield (mg/mg)	Wash
1	Normal	0.63	0.60	Buffer
2	Normal	0.58	0.63	Buffer
3	Normal	0.61	0.54	Distilled Water
4	Normal	0.62	0.56	Distilled Water
5	Normal+	0.62	0.46	Buffer
6	Normal+	0.58	0.47	Buffer
7	Normal+	0.60	0.47	Distilled Water
8	Normal+	0.59	0.46	Distilled Water

Abbreviations: Normal+, minimal medium with 750 mg/l of ammonium sulfate instead of 500 mg/l and 200 mg/l of magnesium sulfate instead of 100 mg/l.

CHAPTER IV

DISCUSSION

An energy requirement for maintenance of cells beyond the requirement for growth had been postulated as early as 1898. Direct measurement of this energy has been found difficult. Hence, most measurements have been based on indirect methods. Neither approach has produced an accepted general procedure. The use of continuous culture has resulted in a more convenient method of estimating maintenance energy. The studies reported herein employ this system for measuring yields which in turn reflect indirectly upon the maintenance requirement. The results of the yield studies represent the prime focal point of this research. Interpretation of the results must necessarily include the maintenance theory although measurements of this quantity can only be estimated from the results herein.

The results of Gaudy and Srinivasaraghavan (1974) working with a mixed culture prompted the present studies. Their findings showed that mixed cultures grown in either a once-through chemostat or a cell recycle system result in yields determined by the specific growth rate at which they are grown. This observation has been made by a number of investigators (Abbott and Clamen, 1973; Schulze and Lipe, 1964; Herbert, 1958; et al.). The unique feature of the studies by Gaudy and Srinivasaraghavan was the investigation of yield in batch using inocula from the continuous flow reactors at various specific growth rates. They found

that the yield in the batch experiments was the same as the yield in the continuous flow even though the specific growth rate was much higher in the batch flasks. This experiment proved to be repeatable and was explained on the basis of natural selection. Species with different yields were postulated to predominate at different specific growth rates. The present results utilizing a pure culture of E. intermedia were designed in a similar manner. A once-through chemostat was used to grow a continuous culture of the test organism. Yield analyses showed the same generally observed effect of specific growth rate upon the yield. At a dilution rate of 1/6 hr^{-1} the average yield was 0.38 mg/mg and at a dilution rate of 1/24 hr^{-1} the average yield was 0.32 mg/mg. The same values were obtained for cells grown at either 25° or 30°C. This effect may be explained by the maintenance theory. Since the amount of energy used for maintenance of the cells is presumably constant, the proportion used at lower specific growth rates would be expected to be higher and hence yields would be less. Yields measured at higher specific growth rates would be expected to result in higher yields because the proportion of available energy used for maintenance would be less. It is conceivable that maintenance energy is not constant for all culture conditions. In fact, it is assumed that at high temperatures, high pH or anaerobic conditions the culture may have a higher maintenance energy. This has been the most common explanation for the variation of yields under these conditions. The yields obtained in the present experiments are somewhat less than those commonly reported for E. coli although Monod (1942) reported a constant yield of 0.23 mg/mg. Schulze and Lipe (1964) have theorized that the nutrient medium used by Monod was incomplete and that other limiting factors besides the glucose were present. They measured

yields of 0.44 for a specific growth rate of 0.06 hr^{-1} and 0.54 at a specific growth rate of 0.66 hr^{-1}. These specific growth rates represent approximate dilution rates of 1/17 hr^{-1} and 1/1.5 hr^{-1} respectively. It should be noted, however, they based their yields upon glucose only. This method of measurement, depending on whether metabolic intermediates are formed, would not result in the same values obtained by COD analysis. It is probably a more conservative method of measuring yield but certainly not as accurate. A comparison of the yields reported in the present study with those of Schulze and Lipe show higher measurements in the latter. The pH, temperature and aeration were identical in both studies. Evidently, this difference can be attributed to differences in species or media. Schulze and Lipe used urea as nitrogen source and ten times more yeast extract in their medium than was used in the medium of the present studies. Apparently, their test organism utilized urea as a nitrogen source although Escherichia is generally believed to lack urease. Either of these differences in media may have resulted in the differences in yields. Schulze and Lipe's experiments were performed at faster specific growth rates which should account for some of the differences. It is important to note that during the studies reported herein the yields unaccountably rose from 0.32 and 0.38 to new values of 0.44 and 0.55 for the 1/24 hr^{-1} and 1/6 hr^{-1} dilution rates, respectively. It appears the test organism either mutated to a higher yield strain or already possessed the ability to use glucose more efficiently and did so for some unexplainable reason.

The batch experiments performed to determine the yield of cells not previously grown in continuous culture resulted in an average yield of 0.42. These cells were grown in batch flasks containing 1000 mg/l of

glucose so that μ_{max} was closely approached. Theoretically, 0.42 should be very close to the maximum yield obtainable based upon the maintenance theory. This measurement was determined before the yield changed to the higher values. It was expected the yield would be higher than the 0.38 obtained at the 1/6 hr^{-1} dilution rate since the specific growth rate at this dilution rate is considerably below μ_{max}. The results confirm this prediction.

Examination of the data from the batch flasks inoculated from the chemostat at either 1/6 hr^{-1} or 1/24 hr^{-1} dilution rates present surprising and unexpected results. The maintenance theory is apparently violated in these results. At each chemostat dilution rate, the corresponding batch reactors resulted in nearly identical yields. The yields in the 1/6 hr^{-1}-inoculated batch flasks averaged 0.38 mg/mg as compared to the identical average from the chemostat. Similarly, the yields from the 1/24 hr^{-1}-inoculated batch flasks averaged 0.33 as compared to 0.32 in the chemostat. Two separate series of batch experiments were performed for each dilution rate. The same dilution rate effect upon batch yields was noted in the high-yield culture as well. An examination of the literature for reports of similar effects involving a pure culture proved fruitless. Using a mixed culture this effect can be explained by the selection of low yield species. Indeed, the effect in mixed cultures noted by Gaudy and Srinivasaraghavan (1974) was explained as a selection effect based upon past studies (Gaudy, Ramanathan and Rao, 1967; and Ramanathan and Gaudy, 1969). In subsequent studies (unpublished), they have noted that the chemostat yields for the low dilution rates began to increase after 5-7 mean hydraulic detention times which they postulated would appear to be a reasonable

consequence of selection of species. It is more difficult to assume such an effect in the present results. Selection of species could not occur using a pure culture although it is conceivable yield mutants may have arisen during the course of the growth in the chemostat. Since the effect noted was experimentally repeatable, such a mutation would seemingly have to occur in a highly mutable segment of the DNA. Most of the experiments run did not exceed five days for any particular specific growth rate. Several were run at shorter time intervals and the same effect to varying extents was noted. This would indicate an easily acquired ability of the cells to change their inheritable cell yields. It is difficult to conceive a single mutation which would result in an inheritable change in the cell's yield. A similar effect regarding growth rate has been reported by Hughes (1955) and Harrison and Lawrence (1963). By two entirely different methods these two groups were able to show that within a given pure culture there were cells with inheritable differences in growth rates. Harrison and Lawrence postulate that since most cells in nature exist in low nutrient environments, a selection of slow-growing cells may offset the selection of fast-growing cells during the periods of active proliferation. They noted a rapid adjustment by some of the cells from log growth to starvation growth while others were slower in doing so and were selected against. They present no yield data with their results which makes it difficult to directly apply their findings to the results reported in the present studies. No appreciable differences in specific growth rate were noted in batch reactors inoculated from either a chemostat at a dilution rate of 1/6 hr^{-1} or from one at a dilution rate of 1/24 hr^{-1}. However, if it

is possible for an inheritance of different growth rates to occur, it would seem an inheritance of different yields might also occur.

Realizing "cell memory" may have contributed to the effect noted, the sequential batch experiments were performed to ascertain the effect of such transfer on the cell yield. The results seem to support a genetic interpretation of the effect rather than a "cell memory" effect. Hence, it appears the cells pass the trait from generation to generation. rather than use enzymes already present to establish a particular yield. Both the yields and the specific growth rates remained relatively constant through both transfers. The batch inoculated from the 1/24 hr^{-1} chemostat showed no tendency to slide upward in yield as would be expected if only "cell memory" was involved. Several further transfers had they been performed might well have substantiated this theory. However, assuming the unexpected yields are a result of an easily mutated region of the genome, further transfers would have increased the chances of obtaining a back-mutation. Hence, a point would be reached where back-mutation would obscure the actual results. Since the specific growth rates in both batch reactor series were near μ_{max}, it appears a genetic mutation has occurred at both dilution rates in the chemostat. Thus, the inoculum from each dilution rate carried its yield capability to the batch reactors. A series of glucose concentrations was employed in the batch reactors to determine the effect of specific growth rates upon the yields. The different concentrations limited the specific growth rate to different extents so that a range of values could be examined. The yield was found to remain constant no matter what the specific growth rate.

The maintenance theory provides a logical explanation for the differences in yield observed at the two specific growth rates in the chemostat. However, it provides no answer to the effects noted in the batch experiments. For the yields to remain the same in batch as in the chemostat, a change in the amount of maintenance energy would have to occur to account for the constancy of the yield at the high specific growth rates attained in batch. An increase in maintenance energy would be required but the reason for this increase is difficult to conceive. Possibly, a correlation between substrate-induced death and maintenance energy can be made. Postgate and Hunter (1962 and 1963) described this effect using A. aerogenes. It was observed that cells grown in glycerol and subsequently starved would die much quicker when placed back in a glycerol medium than if they had been grown in the presence of some other carbon and energy source. They presented no explanation for this phenomenon. Growth in a chemostat represents starving conditions. When the starved inoculum from the chemostat is put into the batch reactors in the presence of excess substrate a condition analogous to the Postgate and Hunter experiment occurs. This change may possibly cause the maintenance energy to rise as well as the specific growth rate. An answer to the question of why substrate-accelerated death occurs may provide an explanation for a rise in the maintenance energy.

An interesting result was obtained from measuring the growth constants. These were determined from a series of batch flasks with media containing different concentrations of glucose. The specific growth rates and yields were determined for each flask and a double reciprocal plot was made for each specific growth rate versus substrate concentration. The batch flasks inoculated from the 1/6 hr^{-1} chemostat

resulted in a calculated K_s of 171 mg/l and a μ_{max} of 0.64 hr^{-1}. The batch series inoculated from the 1/24 hr^{-1} chemostat had a K_s of 133.4 mg/l and a μ_{max} of 0.58 hr^{-1}. These growth "constants" apparently are not constant when the cells are growing at different rates. A determination of K_s and μ_{max} directly from the slant would have been desirable as a basis of comparison. However, no such determinations were made. If these heretofore believed constants are subject to change depending upon the specific growth rate a change in maintenance energy might also be expected. Alternatively, if these changes represent the presence of yield mutants it may be expected that any particular yield mutant has its own growth constants as well as maintenance coefficient.

Maintenance energy has been demonstrated a number of times with the use of the continuous culture technique. It provides an adequate explanation for the differences in yield observed at different specific growth rates. However, its manifestation is not always apparent as has been demonstrated in the present studies. Further attempts at explaining this effect on the basis of the maintenance theory must await new research and techniques for studying maintenance.

Direct attempts at measuring the maintenance energy of E. intermedia in the present studies were not made. Normally the maintenance energy is determined by measuring the yield at various dilution rates (specific growth rates) in continuous flow cultures in which the concentration of the carbon and energy source is growth limiting. The actual value for the maintenance coefficient is determined by using a double reciprocal plot of yield against dilution rate which normally results in a straight line. The intercept at the ordinate is the reciprocal of the growth yield corrected for maintenance. The slope of the line is the

maintenance coefficient in grams of substrate/gram of dry weight/hour. Most cultures have been shown to approximate a straight line. A rough estimate of the maintenance coefficient for E. intermedia was made for comparison with those reported in the literature. Using only the two points obtained from the 1/6 hr^{-1} dilution rate and the 1/24 hr^{-1} dilution rate a maintenance coefficient of 0.025 gm of glucose/gm of dry weight/hour was obtained at both the 25° and 30°C temperatures. Schulze and Lipe (1964) calculated a maintenance coefficient of 0.055 for E. coli. This value was determined from yields based upon utilization of glucose rather than COD and for this reason may be subject to error depending upon the amount of metabolic intermediates present. Pirt (1965) utilizing data compiled by Marr, et al. (1963) for E. coli estimated a maintenance coefficient of 0.07. However, since Marr did not present any yield data, Pirt based his estimation upon an assumed yield for E. coli. Both experiments cited are subject to some error as is the value calculated from the data presented in the present study. These values are within range of the maintenance coefficients calculated for other bacteria although they tend to be somewhat lower. For example, Pirt (1965) has reported a value of 0.094 for A. cloacae grown under aerobic conditions and with glucose as the substrate.

The discussion presented has centered upon mutation and alteration of the maintenance requirement as possible explanations for the chemostat-influenced batch yield effect. Studies by Senez (1962) with A. aerogenes using different temperatures resulted in the concept of uncoupling. It is thought that there exists in growing cells an effective coupling between energy-yielding metabolism and the energy-consuming reactions. However, according to Senez, this coupling depends

upon culture conditions. He noted that with A. aerogenes grown between 37° and 42°C an uncoupling occurred between oxidation and growth. Other investigators have observed this phenomenon resulting from other culture conditions such as composition of the culture medium (Hernandez and Johnson, 1967; and Stouthamer and Bettenhaussen, 1972). The test organism, E. intermedia used in the present studies apparently maintained tight coupling when grown at both 25° and 30°C since the yields remained the same. However, because less substrate was used at 25°C, an effect on the transport system for glucose may have occurred.

Although uncoupling did not appear to occur at either temperature, its possible role in the batch studies is discussed later. Hence, the uncoupling concept provides a third explanation for the effect noted in the present studies. The effect of immediate changes in specific growth rate has been shown to cause uncoupling in A. aerogenes (Tempest, et al., 1967 and Jensen and Neidhardt, 1969). These experiments were performed in chemostats and specific growth rate was varied by changing the dilution rate. It was noted by Tempest, et al. (1967) that bacterial dry weight decreased although oxygen uptake increased when the dilution rate in a glycerol-limited chemostat was changed from 0.003 to 0.24 hr^{-1}. The significance of this observation is unclear since the decrease in dry weight can be attributed to the washout of cells. However, an increase in oxygen uptake probably indicates uncoupling is occurring. An analogous situation can be postulated for the effect noted in the present studies. Removing cells from a slow-growing continuous culture into a fresh medium with excess carbon and energy source may result in inefficient utilization of the substrate for growth. One can visualize the immediate activation of uptake and energy-producing

pathways. If it is assumed the biosynthetic pathways are not activated as quickly as the energy-producing pathways, an explanation for the low yields at high specific growth rates is presented. Although the specific growth rate increases, much of the energy used for assimilation is dissipated until tight coupling is reinitiated. When natural conditions involve high substrate levels, those bacteria with faster growth rates would be expected to predominate. Hence, it appears that under conditions of excess substrate, the cells may prefer a higher specific growth rate to a higher yield when conditions dictate a choice. Marr, et al., (1963) showed with E. coli that yields progressively increased in order from slowly fed cultures to batch cultures to rapidly fed cultures. His yields were based upon optical density. The low yields obtained from slowly fed cultures can be explained on the basis of a higher proportion of the energy used for maintenance. The higher yields obtained with rapidly fed cultures can also be explained by the maintenance theory but the yields in batch can not be. Pirt (1957) and Nilson (1960) have shown an increase in yield as a result of glucose limitation as opposed to the yields obtained in batch where the substrate is in excess. However, based upon the uncoupling hypothesis, this may not be a direct effect of glucose limitation but rather the yield in batch may be lower because of uncoupling in the presence of excess substrate which results in less efficient utilization of substrate. Previous specific growth rate may determine the extent of uncoupling when the cells are placed in a medium containing excess substrate. For example, at a very high previous specific growth rate uncoupling is near a minimum when the cells are placed in a batch medium. Consequently, the yields should be higher if this theory is correct. This type of

experiment has not been reported in the literature. The sequential batch studies reported herein showed no increase in yield through two batch transfers. On the basis of this finding, it appears uncoupling is probably not a factor in the observed effect, although it can not be completely discounted.

As indicated earlier in the results section, the overall cell yield of the test organism unexplainably increased after the major portion of these studies was completed. The same types of experiments were performed with the higher yield cells to determine if growth in the chemostat still affected the batch yields in the same manner. The same effect was shown as a result of these studies and subsequent experiments were designed in an attempt to explain the appearance of the higher cell yields.

During a yield analysis experiment with the higher yield cells, it was observed that cells from the chemostat, when plated on EMB lactose agar plates as a test for purity, resulted in two distinct colony types. These were labelled R and S and are described in Chapter II. To determine if the higher yield was a result of the presence of the cells producing the S colonies, a series of experiments was designed. The results presented in Chapter III are inconclusive regarding the effect of the S type on the yield. However, several findings were interesting and may explain in part the rise in yield. The batch yield studies resulted in nearly equal yields for the R and S culture types. However, a trend was noted in these results. Over a series of 6 experiments done in triplicate for each colony type, the average yields for R were consistently higher than the yields for S. The statistical significance of this observation was not determined. It is important to note that the R

colony type developed into the same type of colonies that typified the test organism of the earlier studies. Since the yield of the R type was the higher of the two, the contribution of the S type to the higher yields is doubtful. The specific growth rates of the two were approximately the same in batch. Figure 13 shows the effect of continuous culture on a mixed culture of the two types. The R type predominated at the 1/6 hr^{-1} dilution rate. When the dilution rate was changed to the 1/24 hr^{-1} dilution rate a rise in the numbers of the S type was observed. The experiment was terminated due to clogged feed lines before it could be determined if the final plate count of the S colony type reached its initial inoculation numbers. These results can be explained on the basis of specific growth rate differences if it is assumed that for some reason the S culture is unable to attain a high enough specific growth rate to prevent wash-out. From the specific growth rate determined in batch, this seems unlikely since it is apparently able to grow at a much faster rate than that in the chemostat. Possibly some interaction between the two cultures results in the predominance of the R type. For example, a compound may be released into the medium by the R type which inhibits the growth of the S type. Alternatively, the R type may be lysogenic. Hence, the R culture would be immune but the S culture would presumably be sensitive if this theory is correct. This may account for the rapid decrease in numbers of the S type. It would also explain the slow rise in numbers of S when the dilution rate was changed to 1/24 hr^{-1}. Resistant cells would be expected to occur and gradually increase in numbers. This theory was untested because the conversion of the S type to the R type prevented maintenance of a pure culture of S. However, before the S culture became

unstable, phage sensitivity experiments were performed. The results shown in Table XI indicate there were definitely differences in the phage receptor sites of the two cultures. Other than colony morphology, this was the only difference that could be determined between the two. Of the 8 phages isolated from sewage, 6 were shown to be specific for either R or S. Hence, it was established that there were differences in the two types but neither could be implicated in the higher cell yields. The most probable explanation is that a mutation occurred yielding a culture with an inherent higher yield.

An interesting observation noted in the chemostat studies of the R and S cultures was the higher total plate counts obtained at the 1/24 hr^{-1} dilution rate compared to the counts at the 1/6 hr^{-1} dilution rate. Although the optical density was less for the 1/24 hr^{-1} dilution rate it appears more cells were present. Ecker and Schaechter (1963) have related cell size to growth rate using Salmonella typhimurium. They showed that cell size was larger at high growth rates but much smaller at low growth rates. Both batch and glucose-limited chemostat cultures were examined.

A final segment of these studies was performed for assurance that the magnesium and nitrogen levels in the medium were not limiting growth. Also, the washing technique was examined to determine if the yield was being affected by washing with distilled water. The results showed that at higher concentrations of magnesium or ammonium sulfate the yields tended to decrease. Stouthamer and Bettenhaussen (1973) have noted that the ionic composition of the medium affects the maintenance coefficient and should likewise affect the yield. The results noted may have been

the result of a similar effect. More importantly, these results showed that the concentrations of magnesium and ammonium sulfate used in all the yield studies were not limiting growth in any way.

The results of the comparison of yields obtained from buffer-washed cells and distilled water-washed cells shows no difference when the cells were grown in the medium containing extra magnesium and nitrogen. In the normal medium, a higher yield was obtained when the cells were washed with buffer. This could possibly be attributed to osmotic shock although more data are desirable before such conclusions are reached.

CHAPTER V

SUMMARY AND CONCLUSIONS

Substrate-based yields in E. intermedia were shown to vary depending upon the rate at which the cells were grown. The faster the cells were grown, the higher the yield. This finding was explained as a manifestation of the maintenance requirement. Similar observations in the literature were pointed out. A unique observation for pure culture was presented. It was found that growth of cells in a chemostat at different dilution rates somehow programmed yield characteristics into the cells. Cells from two different dilution rates were shown to have two different yield values which remained the same when inoculated into batch flasks with excess substrate (1000 mg/l). The near maximum specific growth rate attained by both sets of inocula showed no effect on the yield of either. The stability of the yields was shown by inoculating a new series of batch flasks from the batch flasks which had just completed their growth phase. The yields remained the same through this second growth cycle. This apparent violation of the maintenance theory was observed for the first time in a pure culture. As such, the literature provided little direct aid in explaining the results. However, several theories were presented in an attempt to account for this unique observation. The first was based upon mutation. It was theorized that yield mutants selected for in the chemostat resulted in the observed effect. The second predicted a change in the maintenance coefficient

either in the chemostat or in batch but definitely influenced by dilution rate. The final theory was based upon the uncoupling concept. It was postulated that the immediate introduction of cells into a medium allowing a much higher specific growth rate than that in the chemostat introduced a prolonged uncoupling of energy-producing and energy-consuming reactions which lasted through at least two growth cycles. Each of the theories has its merits as well as its demerits and all of them are possible until further research eliminates or verifies them unequivocally.

Several minor observations were also noted in this study. It was shown that a pure culture of E. intermedia is somewhat heterogeneous. Two different colony types were shown to coexist in the culture. Attempts at completely eliminating either of the two were unsuccessful. It was also found that the only difference between the two were the phage receptor sites of each although more extensive tests may have uncovered other differences. Furthermore, it was shown that neither by itself caused the high yields noted in the latter parts of these studies.

Finally, it was shown that washing E. intermedia with either phosphate buffer or distilled water probably does not significantly affect the yields although further study is warranted.

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VITA

Crosby Wilson Jones, Jr.

Candidate for the Degree of

Master of Science

Thesis: EFFECT OF SPECIFIC GROWTH RATE ON YIELD OF ESCHERICHIA INTERMEDIA GROWING IN BOTH CONTINUOUS AND BATCH SYSTEMS

Major Field: Microbiology

Biographical:

Personal Data: Born at Claremore, Oklahoma on August 1, 1950, the son of Crosby W., Sr., and Mary Jane Jones. Married Janet Keasling, May 26, 1973.

Education: Graduated from Claremore High School, Claremore, Oklahoma, in 1968; received the Bachelor of Science degree in Microbiology from Oklahoma State University, Stillwater, Oklahoma, in May, 1972; completed the requirements for the Master of Science degree in December, 1974.

Professional Experience: Graduate Teaching Assistant, Department of Microbiology, Oklahoma State University, September, 1972 to present; Environmental Protection Agency Bioengineer Trainee, Department of Civil Engineering, Oklahoma State University, June, 1973 to present.