GROWTH DYNAMICS OF MICROORGANISMS

by

James Wade Gilley, B.S., M.S.

Dissertation submitted to the Graduate Faculty of the

Virginia Polytechnic Institute

in candidacy for the degree of

DOCTOR OF PHILOSOPHY

in

Civil Engineering

January, 1966

Blacksburg, Virginia

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

At the present there are only a few large-scale, industrially important, single-stage continuous fermentations in operation. However, biological waste treatment processes such as the trickling filter, activated sludge units, and lagoons are common. In the trickling filter, sewage is applied to a support material, on which a mixed culture of micro-organisms is maintained to digest organic material as it flows through the layers of stone. In the activated sludge process some of the sludge, a biomass consisting of a variety of microorganisms, is recirculated from the down-stream end of the tanks back to the inlet end to serve as a massive inoculum for the incoming sewage. Both of these processes are extremely efficient when they are operating properly, but there are times where these processes are completely unpredictable. Elucidation of the fundamental biology is essential before a meaningful approach for improving complex biological processes can be formulated. Mixed cultures studied by batch techniques tend to give results that are extremely difficult to interpret because of simultaneous changes in such factors as cell number and age, pH, nutrient concentration, waste product accumulation, and the like. Therefore, almost any research into the mechanisms and dynamics of mixed cultures should be done in continuous culture. Generally, continuous flow procedures cause the establishment of steady states which hold constant such environmental factors as nutrient concentration, average physiological age, and physiochemical conditions. Also, continuous culture studies are of additional

interest because the biological treatment processes are essentially continuous mixed cultures.

Shindala (1964) initiated a comprehensive study of the interaction of S. cerevisiae and P. vulgaris. However, the results indicated that a thorough study of the microbial dynamics of pure cultures in continuous culture would have to be undertaken to interpret data on mixed culture interactions. For example, the fact that abrupt changes in dilution rate gave unusual response in population dynamics indicated that pure culture responses would have to be studied to lay a foundation for explaining mixed culture responses. Since biological waste treatments change from one level of an environmental factor to another level of the same factor frequently, it was decided to investigate the response of continuous microbial systems to abrupt changes.

The yeast S. cerevisiae was chosen because of its size which makes it easy to count in the presence of bacteria. For pure culture dynamics, its large size allowed the use of large non-clogging orifices for rapid electronic counting. Actually the key to any dynamic analysis of microbial systems is electronic counting. A Coulter Counter population measurement can be made about once a minute while plate counts or microscopic counts are practical only a few times a day. Some investigators use a chemical analysis of a constituent related to growth or measure turbidity, but these are less precise and require assumptions not needed for electronic counting. Also, the Coulter Counter allows

determination of precise size distributions during population responses to insure that the results do not reflect anomalies in size of cells. The electronic size distributions were also used to relate average physiological age to average cell size.

Swanton, et al (1962) discussed the status of the Coulter Counter for work with bacteria. However, no mention was made of counting mixed cultures. Shindala (1964) and Jacobson (1964) found that the Coulter Counter was very precise in determining relative populations in mixed culture.

The objectives of the present investigation can be summarized as follows:

1. To investigate the effects of dilution rate on steady-state population and to compare the results with continuous culture theory.
2. To investigate the relation of average cell size, protein nitrogen per cell, and dry weight per cell to growth dynamics.
3. To investigate the relationship between steady-state population and medium concentration, and the relationship between the "wash out" dilution rate and medium concentration.
4. To determine the response of microbial systems to abrupt instantaneous changes in dilution rate, medium concentration, ammonium sulfate concentration, glucose concentration, temperature, and pH.
5. To investigate the feasibility of using sinusoidal forcing for analyzing microbial systems.

6. To determine the time constants for the response of S. cerevisiae to several environmental factors by a frequency response analysis.

7. To investigate the mixed culture interaction of S. cerevisiae and E. coli using a two-stage continuous culture system.

REVIEW OF LITERATURE

Dilution Rate Versus Steady State Population

Herbert, et al (1956) extended the work of Monod (1942) to develop a detailed theory for the continuous cultivation of microorganisms. While certain experimental systems have verified this theory, several authors have presented data that is in contradiction. Also, in a later paper Herbert (1958) discussed several possible deviations from the general continuous culture theory. The two most prominent explanations for deviation were found to be insufficient mixing and carbohydrate buildup. Poor mixing violates a key assumption in deriving the theory, and carbohydrate buildup resulted in a linear relationship between dilution rate and steady-state population. This will be discussed in the section on "Theory of Continuous Culture". Several other types of deviation from the theory have been reported in recent years by different authors but few have been well explained. Fiechter and Ettlinger (1962) obtained a steady-state population versus dilution rate relationship for the yeast S. cerevisiae. However, their data must be questioned as they made the invalid assumption that cell size and dry weight per cell were constant over the complete range of dilution rates.

Schulze (1964) found that the assumption that the yield coefficient is always constant to be false. He formulated a new theory for continuous cultures of microorganisms by extending the work of Herbert and Monod based on the implications of his new experimental data.

Oscillatory Responses of Microorganisms

Finn and Wilson (1954) noticed in their work and in the work of others that continuous growth of a yeast culture induced a cycling of the population in contrast to the usual stable steady-state situation. They accounted for this phenomena by assuming that some of the initial disturbance, such as a reduction in flow rate, caused the number of cells to increase. As each cell manufactured acid, the pH fell, causing the growth rate to decrease. Before long there would be a net washout of cells and decrease in population, a sequence of events which would result in steady oscillation if there was a time lag in the response to pH.

To describe the behavior of the yeast population, Finn and Wilson integrated the common balance equation to form a possible mathematical basis for the cycling. From the formulas derived they were able to conclude that this cycling theory would be valid if there was a time delay in the adjustment to pH. Also, Finn and Wilson, were able to deduce that in this cycling the logarithm of the population fluctuated sinusoidally.

Yasuda and Mateles (1964) have demonstrated with E. coli in continuous culture that there is a sudden jump in growth rate following an increase in dilution rate and that the growth rate drifts slightly above the new steady-state value before completing its adjustment. This behavior is interesting and is similar to data in the present investigation but cannot be termed oscillatory.

Herbert (1962) has reported that Monod's equation applies to the unsteady state with less than a 30 second lag. That is, cells can almost instantaneously adjust their growth rate when abrupt changes are made either in the dilution rate or in the concentration of limiting nutrient in the incoming medium.

A variety of biochemical reactions have been put into oscillation by abrupt changes in certain factors. Chance and Schoener (1964) reported that cyclic and oscillatory responses have been observed of the DPNH of reduced pyridine nucleotide and ofother respiratory coenzymes in various types of yeast cells and cell-free yeast extracts. They reported that these oscillations of reduced pyridine nucleotide can be induced by the introductionof glucose to highly aerated yeast cells or cell-free yeast extracts. A symposium was recently held on oscillatory biochemical phenomena (Chance 1965).

Frequency Response Analysis of Biological Systems

Dynamic systems analysis obtains a mathematical description of a system by analyzing its responses to applied disturbances. Such techniques are widely used by chemical engineers (32, 34, 42) for process systems analysis, but there seems to have been no effective effort to apply these techniques to biological systems. Fuld, et al (1962) reported some preliminary work concerning sinusoidal forcing along with some experimental results.

Gilley and Bungay (12), based on work in this thesis, reported preliminary experiments with the yeast S. cerevisiae that indicated that sinusoidal forcing was feasible. Also, time constants for the

present investigation were included for the response of S. cerevisiae to change in dilution rate. The time constants were obtained by use of the Bode Diagram.

Two-Stage Mixed Culture Systems

A continuous two-stage system for investigation of interactions between two or more species of bacteria grown in mixed culture was described by Parker and Snyder (1961). The system employed two chemostatically limited stages of pure culture feeding to mixed continuous growth vessel. At any point on the growth curve, numbers and metabolic products of organisms in pure culture could be compared with data from these same organisms growing in mixed culture. The growth data were expressed as generation times. Results obtained with a mixture of Streptococcus salivarius and Veillonella alcalescens indicated a distinct interaction between the strains employed. Generation time of each of these species was greatly increased while growing with the other.

Shindala (1965) found that in a one-stage continuous mixed culture system E. coli invariably caused the "washout" of S. cerevisiae. He concluded from those preliminary experiments that this interaction was a competition for limiting substrate. It was evident from these experiments that a two-stage system would be necessary to fully explore the phenomena.

Malek (1961) utilized the multistage fermenter in the study of the physiological state of microorganisms grown in continuous culture. The

multistage fermenter appeared to be a very advantageous tool for this study and some examples were presented. The study of the influence of nitrogen limitation on the culture of Baker's yeast in a two-stage system was also reported in which there was in the first-stage limitation of the carbohydrate and excess of all other nutrients, while in the second stage there was nitrogen limitation; the influence of this limitation on the physiological state of yeasts was also shown.

Coulter Counter Enumeration of Microorganisms

Swanton, et al (1962) discussed the use of the Coulter Counter for determining populations of bacteria; viable and living suspensions of Staphylococcus aureus, Escherichia coli, and Serratia marcescens were counted electronically. Microscopic and plate counts were performed at the same time as the Coulter Counter for controls and comparison. This study indicated that the counting of microorganisms could be performed precisely by the Coulter Counter. Also, this study indicated the possibility of determining the degree of viable cells in a mixture of living and dead cells, and it was implied that the Coulter Counter might be used to count in mixed culture.

Kubitschek (20) showed that a Coulter Counter with a 10-micron orifice would give precise results for counting and sizing of bacteria. Deysson, et al (1963) used the Coulter Counter for the study of yeast, and size distribution curves were presented. Shindala, et al (1965) concluded that the Coulter Counter was very precise and rapid in

determining relative numbers of S. cerevisiae and E. coli in mixed culture.

Recently, Curby, et al (1964) presented a procedure to determine the extent of randomness of the presentation frequency of the pulse population generated by bacteria in a Coulter Counter, and to establish the practical minimum sampling interval for observing bacterial population changes electronically.

An instrument for the recording of cell number has been developed by James and Anderson (1963). Changes were recorded continuously in populations of logarithmic and synchronized cultures of protozoan flagellates. A Coulter Counter was used in conjunction with a counting chamber that was fitted with a flexible polyethylene aperture 75-microns in diameter. This system might be used for following the cell number in chemostats or for control of cell numbers in other types of continuous cultures.

Recently, an autoanalyzer cell counting system has been developed (Technicon Instruments Corporation, Research Park, Chauncey, New York) to count red and white cells and hemoglobin in whole blood. This system could possibly be used to count microbial populations. For cell counting, samples automatically diluted and in suspension, enter the replaceable, disposable continuous flowcell of the counter. As the individual red or white cells pass through a focused light beam, they produce light pulses which are converted into electronic signals. The signal rate gives a quantative measure of the rate at which red or white cells pass through the flowcell. The counting rate is proportional

to the blood cell concentration of the sample. Signals are fed into a visual display, and are presented graphically on the autoanalyzer recorder.

THEORY OF THE CONTINUOUS CULTURE OF MICROORGANISMS

Equations developed by Herbert, et al (1956) based on the classical treatment of Monod (1942) for growth of microorganisms in continuous cultures and the equations of Bartlett (1958), for continuous growth of microorganisms in multiple stage chemostats are important to this research.

Consider microorganisms growing in a completely mixed continuous culture vessel, with the inflowing medium containing a single limiting organic substrate at a concentration S_r. Since the other substrates are present in excess, and assuming that the chemostat is sufficiently aerated to provide an excess of oxygen at all times, the supply of limited organic substrate will be the only growth limiting factor. The variables within the immediate control of the researcher are substrate concentration, pH, temperature, and the flow of substrate (dilution rate) into the chemostat. A complete theory should explain how these variables affect the growth rate of the microorganisms and substrate concentration in the chemostat.

In the culture vessel the cells are growing at a rate described by equation (1) and at the same time being washed away at a rate determined by equation (2).

$$\frac{dx}{dt} = \mu x \qquad (1)$$

$$\frac{dx}{dt} = -Dx \qquad (2)$$

Where x is the concentration of organisms in the chemostat (dry weight or number of cells per unit volume at time t), μ is the specific growth rate (the rate of increase/ unit of organism concentration "$\frac{1}{x}\frac{dx}{dt}$" is called specific growth rate), and D is the dilution rate (the number of complete volume changes per unit time or the flow rate divided by the volume of the chemostat). Therefore, the material balance within the culture vessel can be described by:

Increase = growth - output

$$\frac{dx}{dt} = \mu x - Dx \qquad (3)$$

Therefore, if μ is greater than D, the growth rate is positive and thus the concentration of cells will increase, but if μ is less than D the population will decrease, toward zero, thus the culture will be "washed out" of the chemostat. If μ is equal to D, $dx/dt = 0$ and the population is constant, then the culture reaches a "steady-state", with specific growth rate, μ, being exactly equal to the dilution rate D. This equation by itself gives no insight into the prediction of what dilution rate makes a steady state possible. For a complete theory the effect of the dilution rate on substrate concentration in the chemostat must be considered.

The substrate is entering the chemostat at concentration S_r, with a certain portion being utilized and the remainder leaving at concentration S. The net rate of change in substrate concentration can be expressed as:

Increase = input - output - consumption (4)

Originally Monod (1942) developed a simple relationship between growth and utilization of substrate. Monod assumed that Y, the yield factor is constant, but Schulze (1964) has shown that the yield factor is definitely not constant. The variance of Y (which is the ratio of the weight of bacterial cells produced to the weight of substrate consumed per unit time) can be established at a series of steady states. If F is equal to the flow rate we can express the substrate consumed (S_c) as:

$$S_c = F(S_r - S) \tag{5}$$

and since the amount of cells produced can be expressed as

$$X = Fx \tag{6}$$

we can express the yield factor as

$$Y = \frac{Fx}{F(S_r - S)} = \frac{x}{S_r - S} \tag{7}$$

From the Schulze experiments one can conclude that at high growth rates substrate is more effectively converted to cell tissue.

The rate of substrate consumption at various specific growth rates can readily be calculated from the following equations:

$$\mu_s = \frac{FS_r - FS}{Vx} = \frac{DS_r - DS}{x} \tag{8}$$

where μ_s = specific rate of glucose, uptake is mg per gram cell weight per hour.

V = volume of chemostat.

A plot of μ_s versus D as given by Schultz demonstrates that μ_s can be expressed as:

$$\mu_s = n + hk \tag{9}$$

where n = y intercept, representing grams of glucose uptake per gram cell weight per hour, and h = constant (slope of equation 9).

The constant k is the inverse of the corrected yield constant Y'. The specific rate of substrate removal can be expressed as:

$$\mu_s = n + \mu Y' \tag{10}$$

and the growth rate can be related to the rate of substrate removal by

$$\mu = Y'(\mu_s - n) \tag{11}$$

In the development of continuous flow equations the growth rate is usually assumed to be a constant fraction of the substrate removal rate:

$$\frac{dx}{dt} = - Y \frac{ds}{dt} \tag{12}$$

and

$$\frac{ds}{dt} = - \frac{dx}{dt} \frac{1}{Y} = \frac{\mu x}{Y} \tag{13}$$

A close examination of equation (13) will reveal that it must be altered because of the small amount of substrate consumption which is evident even if no growth occurs so that:

$$\frac{ds}{dt} = - (nx + x\mu_1/Y') \tag{14}$$

$$\frac{ds}{xdt} = \mu_s = - (n + \mu_1/Y') \tag{15}$$

Equation (15) allows the calculation of net yield Y at various levels of μ. Therefore by definition:

$$Y = \mu/\mu_s \tag{16}$$

and thus

$$Y = \frac{\mu}{n + (\mu/Y')} \tag{17}$$

From equation (17) one can see that the actual yield factor will decrease with decreasing growth rate.

Therefore the balance equation (3) can be expressed as:

$$\frac{ds}{dt} = DS_r - DS - x(n + \mu/Y') \tag{18}$$

If we consider the condition where $dx/dt = 0$, steady-state conditions, we can write

$$D = \mu_m (1 - e^{-CS}) \tag{19}$$

where μ_m = maximum growth rate constant, and C = constant

and

$$\bar{S} = \ln(\mu_m/(\mu_m - D))/C \tag{20}$$

where $\bar{S}$ = substrate concentration in chemostat and effluent at steady-state.

Since $\mu = D$ at steady-state

$$\bar{x} = \frac{D(S_r - \bar{S})}{n + D/Y'} = \frac{Y'(S_r - \bar{S})}{nY'/D + 1} \tag{21}$$

Where $\bar{x}$ = cell concentration in chemostat at steady-state conditions.

The above equations define the cell and substrate concentrations for steady-states ranging from $D = 0.02$ to $\mu = \mu_m$.

Also, we are interested in determining the critical value of the dilution rate where complete washout occurs. This value of dilution rate, D_c, is equal to the highest possible value of μ, which is attained when $S_r = S$. This value is:

$$D_c = \mu_m(S_r/\mu_s + S_r) \tag{22}$$

In recent years there have been several reported deviations from the general continuous culture theory. Herbert (1958) discussed possible deviations from the theory and concluded that carbohydrate build-up and insufficient mixing were the major problems in the data reviewed. Insufficient mixing was a deviation from the theory and produced a critical dilution rate, D_c, that was greater than the theoretical critical dilution. Carbohydrate build-up resulted in a steady-state versus dilution rate curve that decreased almost linearly as the dilution rate increased.

In 1953, Golle showed that it is sometimes desirable to operate a continuous culture with the effluent of the first chemostat flowing continuously as feed to a second chemostat, which may flow into more culture vessels if desired. There are certain advantages in the use of multiple stage chemostats in both fermentation and mixed culture experiments. In industrial fermentations, by proper adjustment of growth rates one may attain a total retention time the same as a single stage chemostat with a more complete use of the substrate. Parker and Snyder (1961) have used a continuous two-stage system for investigating interactions of two or more species of bacteria grown in mixed culture. In analyzing the theoretical behavior of such a system Equation (4) can be applied while the second chemostat is being filled giving:

$$FX_1 + \mu_2 XV = V dx/dt \qquad (23)$$

where X_1 = concentration of cells coming from the first chemostat.

X = cell concentration in the second chemostat

V = volume in second chemostat at any time

rearranging we obtain:

$$dt = \frac{d(VX)}{FX_1 + \mu_2 VX} \tag{24}$$

integration gives

$$t = \frac{1}{\mu_2} \ln((FX_1 + \mu_2 VX)/FX_1) \tag{25}$$

When $t = t_r$ (i.e. when the vessel begins to overflow at V_2/F)

$$VX = V_2X_2 \tag{26}$$

and equation (26) becomes

$$FX_1 e^{(\mu_2 V_2/F)} = FX_1 + \mu_2 V_2 X_2 \tag{27}$$

at steady-state, when $V_1 = V_2$, $F/V_2 = \mu_1$ equation (27) reduces to

$$X_1 e^{\frac{\mu_2}{\mu_1}} = X_1 + \frac{\mu_2 X_2}{\mu_1} \tag{28}$$

which gives

$$X_2 = \frac{\mu_1 X_1}{\mu_2} (e^{\frac{\mu_1}{\mu_2}} - 1) \tag{29}$$

The maximum value of X_2 occurs when $\mu_1 = \mu_2$ since μ_2 cannot exceed μ_1. For this condition

$$X_2 = X_1 (e - 1) \tag{30}$$

$$X_2 = 1.718\ X_1 \tag{31}$$

Therefore it becomes apparent that the concentration of organisms in the second chemostat should in theory never be more than 1.718 times higher than in the first chemostat at the moment overflow begins.

Next we should consider the continued overflow from the second vessel after it has achieved steady state; this would follow the theory for a single vessel with recycling. That is

Input + Growth = Output + Accumulation

$$FX_o + \mu XV = FX + V\frac{dx}{dt} \tag{32}$$

$$\frac{dx}{dt} = D(X_o - X) + \mu X \tag{33}$$

$$\frac{dx}{dt} = DX_o + X(\mu - D) \tag{34}$$

When $\mu_2 - D_2 > 0$

$X_2 \longrightarrow \infty$

as $t \longrightarrow \infty$

and when $\mu_2 - D < 0$

$$\lim_{t \to \infty} X_2 = \frac{D_2X_1}{D_2 - \mu_2} \tag{35}$$

also when $\mu_2 - D_2 = 0$

$$X_2 = D_2X_1t + X_1 \tag{36}$$

so that $X_2 \longrightarrow \infty$ with increasing time.

Contamination effects

Contamination can be a very serious problem in continuous cultures since the long periods of time for which continuous operations are designed can make them particularly susceptible to the introduction of unwanted organisms. The theoretical behavior of contaminates was expressed by Golle (1953) and the following assumptions were made:

1. The medium entering the culture vessel is the only source of contamination, and this medium contains X_o number of cells per volume V_o.
2. The culture vessel is operating under steady-state conditions; therefore $R/V = D = \mu$.

for these conditions the following equation exists:

$$\frac{dx}{dt} = DX_o + \mu_c - DX \tag{37}$$

where μ_c = growth rate constant of the contaminant.

Thus by integration we obtain:

$$\frac{X^c(\mu_c - \mu) + \mu X_o^c}{X_1^c(\mu_c - \mu) + \mu X_o^c} = e(\mu_c - \mu)t \tag{38}$$

As discussed before, three possibilities exist with respect to the growth rate of the contaminant cells in the continuous culture. That is, μ_c can be greater than, equal to, or less than the dilution rate D. If $\mu_c > D$ then X^c will increase exponentially until a steady-state is achieved and $\mu_c = D$. Under these conditions the growth of the original organism will be less than D and will be washed out. After a period of time the original organism will be entirely replaced by the contaminating organism. This will also happen if $\mu_c = D$ and its population increases linearly at a rate DX_o^c. However, if $\mu_c < DX_o^c$ will approach a definite limit at

$$t \longrightarrow \infty$$

$$X^c = \frac{DX_o^c}{D - \mu_c} \tag{39}$$

A contamination by such an organism will become serious if its rate of entry is extremely high and its growth rate only slightly less than that of the desired organism.

ELEMENTARY DYNAMIC ANALYSIS THEORY

Dynamic systems analysis obtains an exact mathematical description of a system by analyzing the response of that system to an applied disturbance. Disturbances are of four general types: Step, pulse, ramp, or periodic (see Figure 1). Step changes are instantaneous shifts from one input intensity level to another. Pulse changes are sudden surges with a return to the pre-surge intensity level. Periodic changes are variations in intensity that repeat within a fixed period of time. Testing using pulse or step changes is called transient response analysis, while use of periodic changes (usually as a sine wave) is called frequency response analysis. In both techniques, a change is applied to the input signal of a system and is compared to a corresponding change in the system output signal.

Consider an elementary system such as a container with instantaneous and complete mixing with a constant rate of flow into the container and continuous overflow to maintain a constant volume. Since mixing is instantaneous the outflow is the same composition as the contents of the vessel. If the feed is suddenly changed from pure water to a solution of salt, the output concentration will increase as shown in Figure 2 until it ultimately reaches the input concentration. The mathematics of this system can be expressed as follows:

let Q = to the amount of salt at any time t

A = change in salt concentration

F = rate of flow (vol./unit time)

I = initial salt concentration

t = time

then

$$\frac{dQ}{dt} = \text{rate of gain} - \text{rate of loss}$$

$$= AF - FQ/V = -F/V(Q - AV)$$

or

$$\frac{DQ}{Q - AV} = (F/V)\, dt + C$$

integrating gives

$$\ln(Q - AV) = -(F/V)t + C$$

or

$$Q - AV = Ce^{-(F/V)t}$$

Now if q = concentration of salt at any time

$$q = A + (C/V)e^{-t/(V/F)}$$

and if when t = 0 q = I

then

$$q = I + A(1 - e^{-t/\tau})$$

where τ is the characteristic time constant for the system (and is equal to the detention time (V/F) for this particular problem).

This is a step change, and the outflow concentration shows a transient response until it reaches the input concentration. For a more complex system the transient response and the accompanying mathematics are more complicated, but there are simple procedures for the analysis of response to step inputs and up to three time constants can be resolved. The only limitations are that the systems be linear (the differential equations describing the process must not contain powers of the variable).

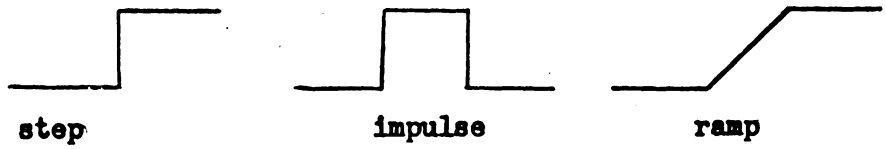


Fig. 1 Typical input functions

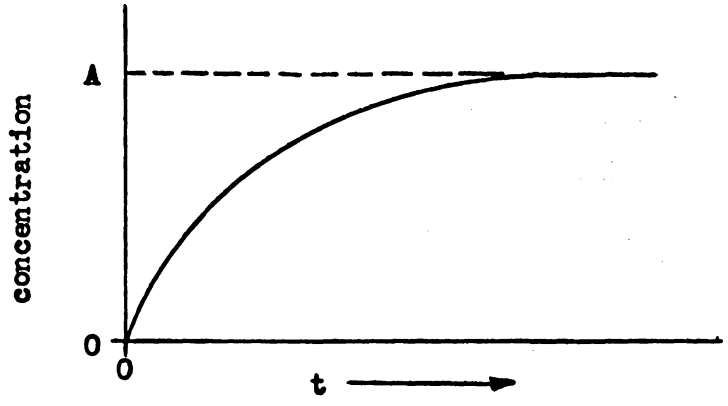


Fig. 2 Typical step response of a first-order system

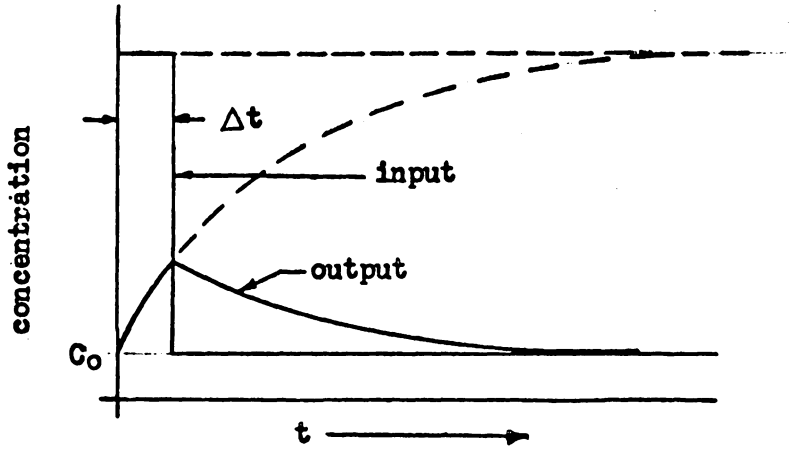


Fig. 3 Typical response of a first-order system to a rectangular impulse forcing

Another method of analyzing systems response is the impulse, which is different from the step in that the duration is brief. The response to an impulse begins in the same manner as for the step change, but the input duration is not long enough for complete response. Figure 3 illustrates a typical response to an impulse. This response may be analyzed for time constants but is limited to fairly simple linear systems.

Since the mathematics of response to step and impulse can quickly become quite complicated, the technique of frequency response to periodic forcing is used for more complex systems. Sinusoidal forcing is usually preferred because it is mathematically simple. To understand frequency response let us again consider the container described previously, but this time the feed is varying sinusoidally about some base concentration, C_o. The output concentration will also vary sinusoidally but may differ from the input in magnitude and in phase. At a given frequency the relationship may be as shown in Figure 4. The ratio of output to input amplitude is termed gain or attenuation. At low frequencies (long sine wave periods), the ratio is close to one, and the phase shift zero. At higher frequencies the holdup in the tank tends to attenuate the output oscillations, and the output cannot follow the input and tends to lag. There are a variety of ways of demonstrating this graphically, but the Bode plot has wide acceptance. Any system described by a first-order differential equation is termed first order. The Bode plot for a first-order system is shown in Figure 5.

Generally the ordinate for magnitude is gain in decibels, but since

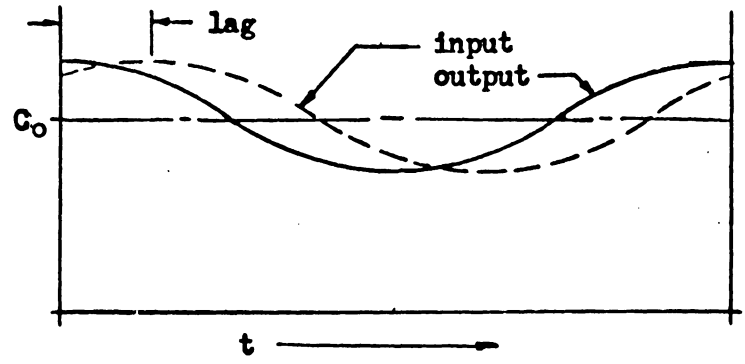


Fig. 4 Typical relationship between input and output in sinusoidal forcing

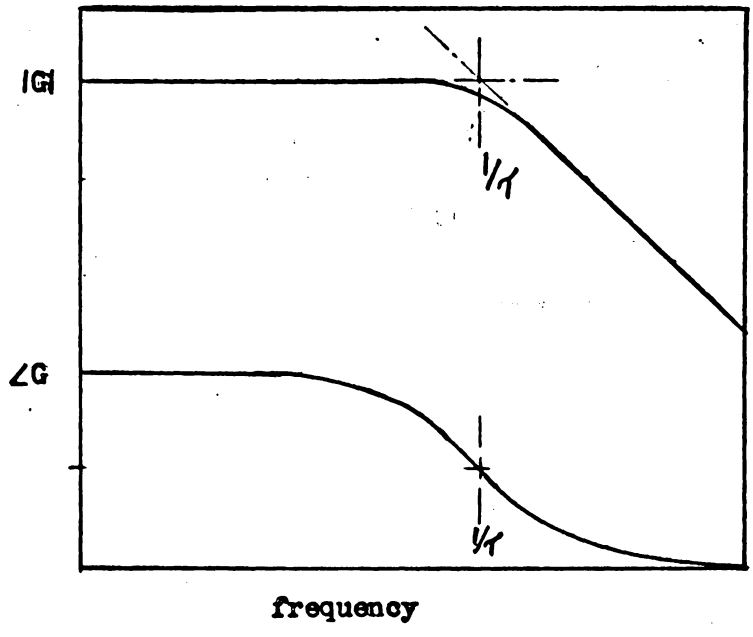


Fig. 5 Typical Bode plot for first-order system

decibels is a holdover from acoustical engineering we will use the ordinate as simply the logarithm of the amplitude ratios. The ordinate for phase lag is in degrees difference in output and input sine wave peaks. The abscissa is in logarithm of cycles per unit time. The Bode Diagram for a first-order system has two straight portions which can be extended to meet at the "corner frequency", and this corresponds to the reciprocal of the time constant. The phase shifts a full-90° for the first order system and has an inflection point as it crosses the -45° line. This inflection point is at the same frequency as the "corner frequency" of the gain curve, thus we have two means of determining the time constant. These two curves will have exactly the same shapes for any first-order system, but will be shifted horizontally to make the time constant agree with the "corner frequency", and vertically for the steady-state gain. If a few good points are obtained experimentally, the rest of the curve can often be sketched by tracing a standard first order Bode plot.

There are several possible Bode Diagrams for second order systems and some of these are illustrated in Figures 6 and 7. Figure 6 is a combination of two first-order systems and the frequency response is the sum of each. At the changes in slope of the gain curve, both time constants can be calculated. The same curve could be obtained by graphical addition of individual first-order Bode Diagrams. Figure 7 is for a true second-order system and indicates that there is a range of frequencies where the input can be amplified (the magnitude ratio

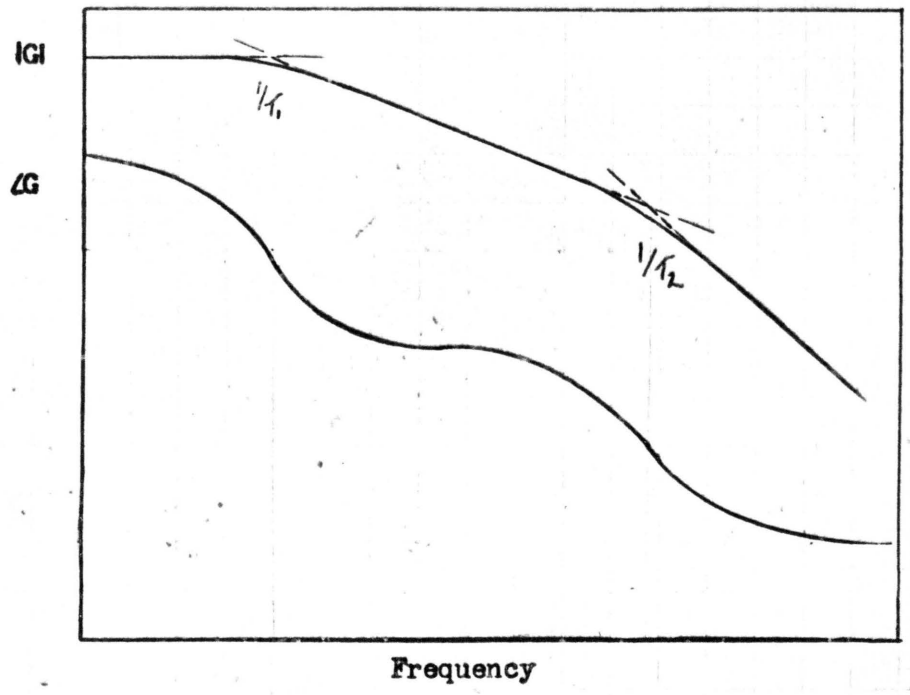


Fig. 6 Typical Bode plot for second-order system

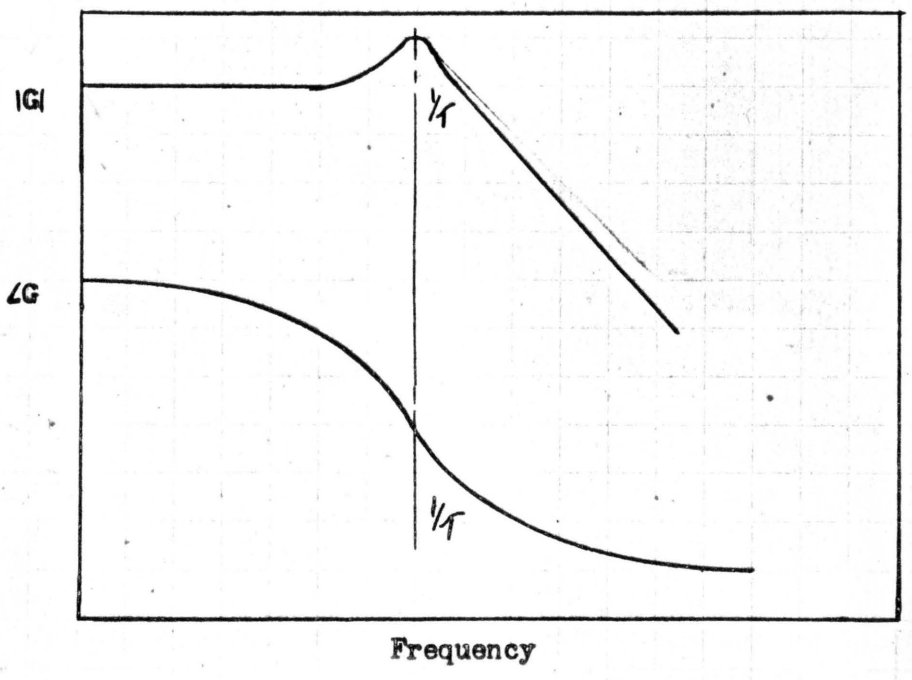


Fig. 7 Bode plot for quadratic second-order system

may be greater than one). The peak response is the critical resonant frequency and again relates to the time constant. The height of the peak depends on the damping factors in the system. Figure 8 is a typical template for a second-order system showing the relationship between the resonant frequency and the damping factors.

The frequency response approach can be used quite easily by the biologist in such problems as control mechanisms, kinetics, mass transport, and population interactions. The frequency response can be used to determine time constants for a biological system, and for microbial cells such time constants could be related to fundamental processes of the following types:

1. Diffusion through the medium
2. Penetration of films
3. Membrane transport
4. Molecular and structural migration within cells
5. Biochemical reactions

In most complex systems one or two time constants will be larger than the others and determine the transient responses. Probably the complex responses of microbial populations would be so dominated, and the processes could be studied in vivo rather than with cell fragments.

If in a biological system that is controlled such as a continuous culture system there are several input signals that can be forced sinusoidally. These could be dilution rate, pH, temperature, or substrate concentration. The output could be growth rate, a rate of a biochemical reaction, a waste product concentration, or the like. Some of

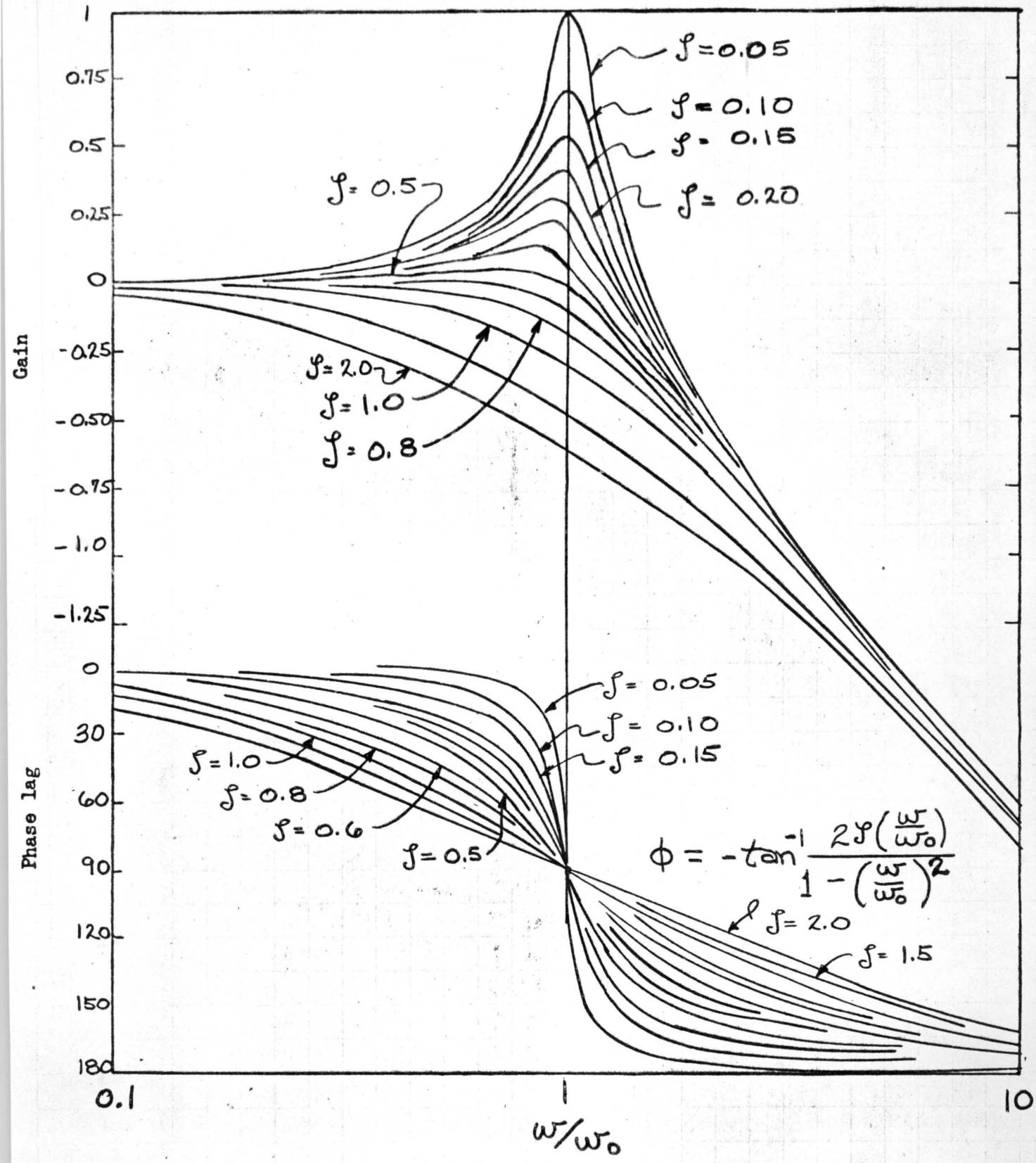


Fig. 8 Quadratic lag template

these input signals are quite easily produced, such as dilution rate and medium concentration, but such variables as pH and temperature require more advanced instrumentation to obtain the feedback control needed.

METHODS AND MATERIALS

A. Description of Continuous Flow Apparatus

In this research three types of continuous culture vessels were employed. A summary of the primary differences is shown in Table 1. Temperature control was obtained with a 30° water bath, and the magnetic mixer was aligned to stir both the bath and the culture vessel. The magnetic stirring bar in each culture flask was one inch long. Air was sterilized by filtering through sterilized cotton. Inoculation of a culture vessel was accomplished by pipetting through a 3/8-inch stainless steel entry pipe capped with a rubber stopper.

The medium reservoir was a 12-liter round bottom flask. A 1/4-inch stainless steel tube in the stopper extended to the bottom and gum-rubber tubing of a selected size connected from the reservoir to the culture vessel. Sigma-motor pumps (Model T8, Middleport, New York) were used to pump the media continuously at chosen rates of flow. Flow rates were established by the size of tubing and the selected motor speed. A tube packed with sterilized cotton was used to equalize the media reservoir with the atmosphere.

The most important differences between chemostats Type A and Types B and C were: (1) indentations were placed in Types B and C to aid mixing; (2) a capillary tube was used in Types B and C to insure minimum lag in outflow. These improvements provided a better designed chemostat. The use of hypodermic needles allowed the change of media reservoirs without contaminating the culture vessel.

Chemostat	Aeration	Overflow	Type of Flask	Inflow
A	Air diffuser	Side arm overflow	250 ml. filter flask	Kjeldahl bulb
B	Surface	Capillary tube extending to fluid level	500 ml. wide mouth flask	Hypodermic needle
C	Air diffuser	Capillary tube extending to fluid level	500 ml. wide mouth flask	Hypodermic needle

Table 1. Summary of Culture Vessels

The two-stage system utilized in the mixed culture experiment consisted of two Type A culture vessels connected in series. The overflow from the first vessel was fed into the second vessel. Provision was made to allow a sample to be taken from the outflow of either vessel.

The entire apparatus was autoclaved at 15 psi for 20 minutes before starting the experiment. After a reservoir of media was consumed, the hypodermic needle was removed from the vessel stopper and another sterile media reservoir was attached to the chemostat.

B. Cultures and Their Maintenance

All cultures were obtained from the biology department culture collection, Virginia Polytechnic Institute, Blacksburg, Virginia. Saccharomyces cerevisiae is egg-shaped, variable sized, gram positive, and optimum growth temperature is room temperature. Escherichia coli is a small rod, approximately one micron as an equivalent diameter, gram negative, aerobic and facultatively anaerobic, optimum growth temperature is 37°C. S. cerevisiae was maintained and subcultured numerous times with Sabouraud's agar and Tryptic soy broth. E. coli was maintained and subcultured using Tryptic soy agar and Tryptic soy broth.

C. Composition and Preparation of Media

Two different chemically defined media were used. The first shown in Table (2) was proven to be nitrogen limited, and was used in various dilutions during a major portion of this research. The

Table 2. Composition of the Nitrogen Limited Media Used for the Growth of S. cerevisiae and E. coli (Medium 1).

Part 1	Quantity/100 ml. Distilled Water
Potassium phosphate, dibasic (K_2HPO_4)	3500 mg.
Potassium phosphate, monobasic (KH_2PO_4)	1500 mg.
Sodium citrate 2 H_2O	250 mg.
Magnesium sulfate 7 H_2O	50 mg.
Ammonium sulfate $(NH_4)_2SO_4$	500 mg.
Part 2	
Glucose	1000 mg.

Part 3 (Vitamins)	Quantity/100 ml. of Media
Biotin	10 µg.
Calcium pantothenate (pH adjusted to 6)	1 mg.
i-inositol	5 mg.
Thiamine	1 mg.
Pyridoxine (pH adjusted to 6)	200 µg.

Part 3 was sterilized by filtration and kept as a concentrated stock solution. The final proportions after separate sterilization were:

Part 1 2 liters

Part 2 2 liters

Part 3 plus distilled water to give 10 liters.

second medium is shown in Table (3) and was proven to be glucose limited. The vitamins were sterilized separately, using a Millipore filter and stored in 10 ml. portions, until time of usage. Usually the media (without vitamins) were prepared in stock solutions and stored until needed.

D. Cleaning of Glassware

Because minute quantities of vitamins or inhibitors could cause erroneous stimulation or inhibitation of growth in nutritional work, and dust particles might affect the Coulter Counter counts or plug the small orifice tube, it was necessary to take care in the cleaning of all the glassware. First, the glassware was soaked in a 1:100 solution of 7x detergent (Linbro Chemical Company, New Haven, Conn.) for about 24 hours, scrubbed and rinsed in tap water, and rinsed about 5 times in distilled water. The glassware was then inverted upon paper towels and left in the air to dry. Glass equipment was capped with aluminum foil and stored until use. For the diluted samples for Coulter Counter counts, the beakers were rinsed in saline four times before use.

Pipettes were soaked in 1:100 7x solution for 24 hours and then rinsed with an automatic continuous device for at least one hour in cold soft tap water. These pipettes were sterilized in an aluminum canister by storage in a 200°C oven for at least two hours.

E. Operation of the Continuous Flow Apparatus

The continuous flow apparatus connected to the reservoir was sterilized intact in the autoclave for 20 minutes. The culture vessel was charged by pumping about 100 ml. of medium and inoculated

Table 3. Composition of the Glucose Limited Media Used for the Growth of S. cerevisiae (Medium 2).

Part 1	Quantity/100 ml. Distilled Water
Potassium phosphate, dibasic (K_2HPO_4)	7000 mg.
Potassium phosphate, monobasic (KH_2PO_4)	3000 mg.
Magnesium sulfate 7 H_2O	100 mg.
Ammonium sulfate $(NH_4)_2SO_4$	1000 mg.
Part 2	
Glucose	100 mg.
Part 3 (Vitamins)	Quantity/100 ml. of Media
Biotin	10 µg.
Calcium pantothenate (pH adjusted to 6)	1 mg.
i-inositol	5 mg.
Thiamine	1 mg.
Pyridoxine (pH adjusted to 6)	200 µg.

Part 3 was sterilized by filtration and kept as a concentrated stock solution. The final proportions after separate sterilization were:

Part 1 2 liters

Part 2 2 liters

Part 3 plus distilled water to give 10 liters

Table 3. Continued

Note: Some difficulties were experienced in autoclaving concentrated glucose solutions as the solution turned a dark brown color when autoclaved. It was found that by making the glucose solution as dilute as practical, this problem could be eliminated.

with about 5 ml. of the culture S. cerevisiae. This inoculum was prepared by transferring one loop of the culture from a 24-hour slant to 10ml. of sterilized distilled water. Growth in the vessel was then allowed to proceed batchwise for 14 hours or until there was obvious turbidity. Medium flow to the culture vessel was then started at the selected rate. Samples of the culture were collected at timed intervals and the population determined with the Coulter Counter. Gram stains and pH measurements were made periodically.

A homemade orifice of approximately 30-microns diameter was used to determine the population of S. cerevisiae and E. coli. A commercial 50-micron orifice was used for a major portion of the S. cerevisiae dynamic analysis experiments. Approximate curves, (a plot of the difference in counts versus the average corresponding threshold level), for E. coli and S. cerevisiae were prepared. The abscissa values in these plots were calculated from t' (threshold setting) as d (corresponding equivalent diameter) using formulas supplied by the manufacturer (Coulter Electronics Inc., Chicago, Illinois).

$$d = k(t)^{1/3}$$

$$t = t' \times F$$

Where d is the equivalent diameter, in microns, k is the calibration constant, t' is the threshold setting, and F is a scale factor depending on the aperture setting and resistance between the electrodes. These data were then replotted as shown in Figure 9.

The size distribution of S. cerevisiae at different media concentrations and dilution rates was determined using the 50-micron orifice. Typical size distribution curves are shown in Figure 10. The average equivalent diameter was taken from the mode of these curves.

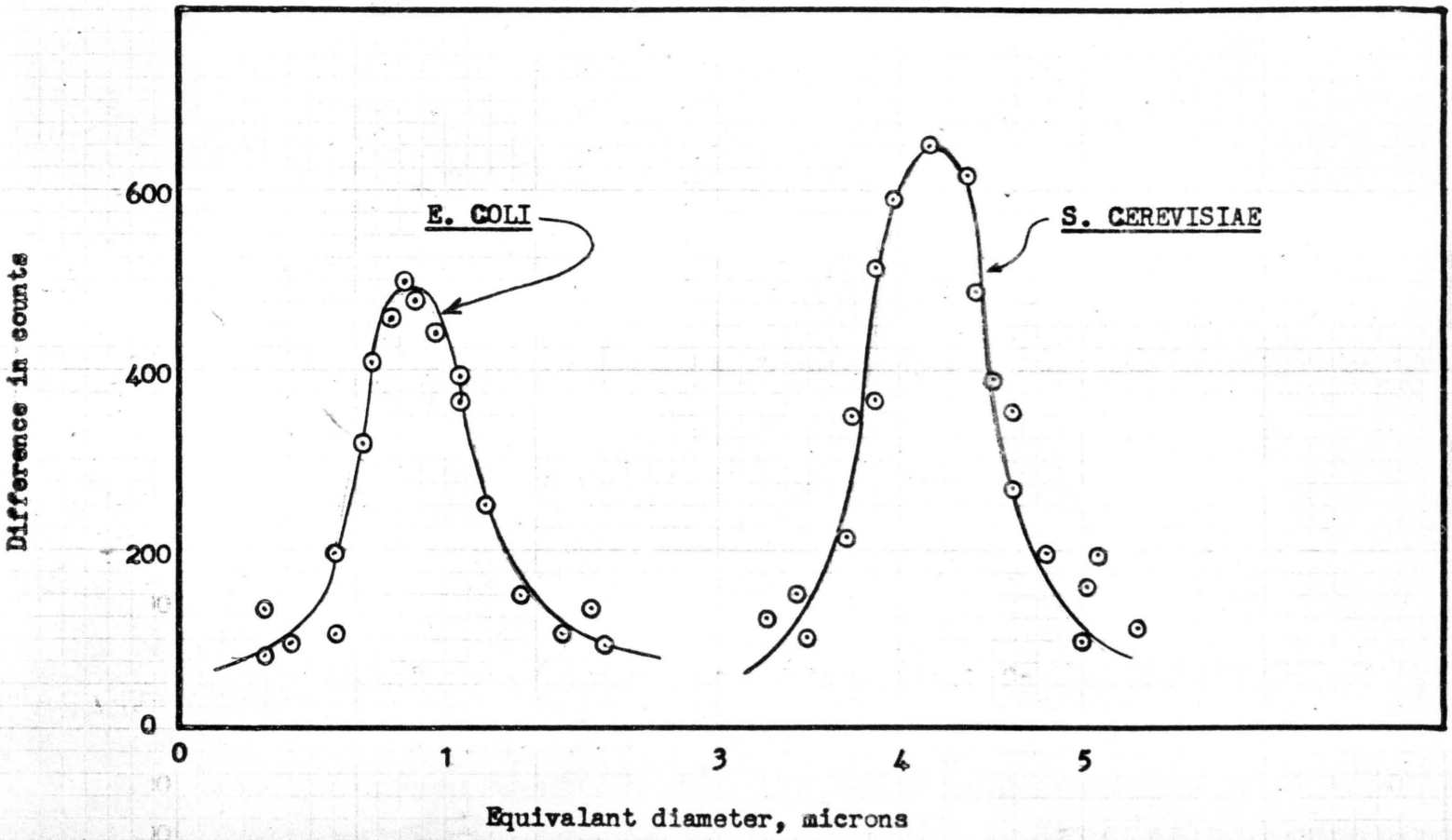


Fig. 9 Size distribution curves for E. Coli and S. Cerevisiae

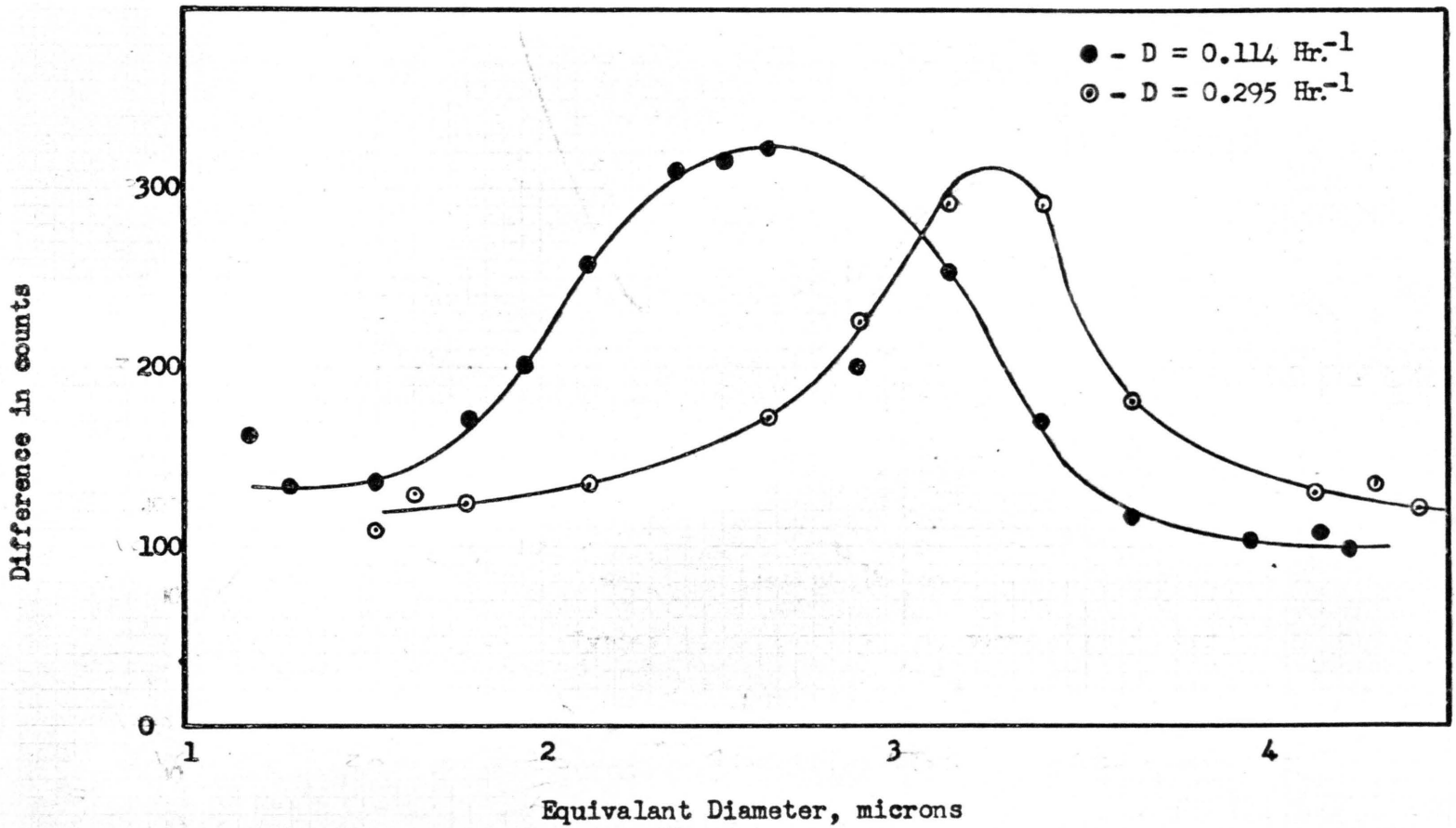


Fig. 10 Typical size distribution curves for S. Cerevisiae at different dilution rates

The sinusoidal input waves for the dynamic analysis experiments were produced by a pulse generator built by Sutherland (1966). This generator consists of an interval timer which sends pulses of variable width to activate pumps so that the composite flow rate closely approximates a sine wave. The timer consists of a drum which rotates continuously at 24 rpm and carries a raised spiral that deflects a mercury switch. At one end of the drum, the switch is closed 100 percent of the time and at the other end it is always open. The percentage of time closed at other positions on the drum is proportional to the distance from the end. The mercury switch is moved back and forth across the drum in a sinusoidal fashion by an eccentric pin in a slot. The selected speed at which the pin rotates gives the period of the sine wave. This period is divided into 24 pulses of varying duration per minute. Thus the number of segments simulating the sine wave is a function of the period. As reported by Sutherland the sine waves were quite satisfactory for frequency response techniques.

Cell dry weight was determined by weighing the solid fraction from centrifuged culture fluid after washing twice by resuspension in distilled water. The Kjeldahl nitrogen assays used conventional digestion and distillation techniques (38). The samples for these assays were washed three times by resuspension in distilled water after first being centrifuged. All dry weight and Kjeldahl nitrogen data points were obtained in duplicate for a check.

F. Analysis of Counting Procedures

There are two common methods of detecting numbers of microorganisms:

one is direct microscopic counting, and the other is by plate counts. Coulter counts were compared periodically with Petroff-Hausser counts and an example is shown in Table (4). On the average, counts obtained by using the Petroff-Hausser chamber were within 20 percent of those obtained electronically. Since the Petroff-Hausser method is very crude this agreement is reasonable. The Coulter Counter is believed to be precise and the order of magnitude is confirmed. For each sample, the method of preparation for electronic counting was the same. A sample slightly greater than 1/2 ml. was collected in an aluminum cup, then 1/2 ml. was pipetted into a 150 ml. beaker containing 99.5 ml. of membrane filtered saline to give a dilution of 1:200. The clean beaker was rinsed at least two times with saline immediately before use. The number of rinsing times before reuse of the same beaker was determined experimentally. Table (5) shows the effect of rinsing on Coulter Counter background counts. The beaker and beaker stand were placed in a closed box with filtered air flowing through as a means of reducing the possibilities of contamination with dust in the atmospheric air. Plugging of the orifice was never a serious problem during this research.

Counts of S. cerevisiae were deducted from those including E. coli to determine the number of E. coli. Background counts were never greater than 200 for all counts quoted, and threshold settings of less than three were not needed. The Coulter Counter settings for counting S. cerevisiae and E. coli are not presented as a homemade orifice or undetermined size was used for this work. However, the settings for the 50-micron orifice used for the dynamic analysis counting are

Table 4. Comparison of Coulter Counts with Petroff-Hausser Counts of S. cerevisiae.

Example	Coulter Counter	Petroff-Hausser
1	12.7×10^6	14.2×10^6
2	10.2×10^6	11.5×10^6
3	8.7×10^6	10.2×10^6
4	15.2×10^6	17.8×10^6
5	9.3×10^6	12.2×10^6

Table 5. The Effect of Rinsing of Beakers on the Coulter Counter Background Counts.

Example	Number of Rinsings	Background Count
1	0	652
2	1	287
3	2	127
4	3	112
5	4	122

presented in Table (6). All the orifices used in this research were calibrated using latex spheres 1.305 microns in diameter supplied by Dow Chemical Co., Midland, Michigan, and for each orifice used calibrating factors were determined. Calibration was performed following the procedure outlined in the instruction manual supplied by the manufacturer.

To evaluate the accuracy of the Coulter Counter, a large sample of cells was divided between four different beakers in different amounts and counted electronically. Then the counts were converted to number per ml. of original sample for each beaker. The results shown in Table (7) show that the size of sample has little effect on the accuracy of the counter.

Table 6. Coulter Counter Settings for Counting S. cerevisiae with a 50-Micron Orifice.

Threshold (t')	5
Aperture Current (I)	7
Gain Index (G)	3

Table 7. Comparison of Sample Size on Coulter Counter Counts.

Beaker	Sample Size	Dilution	Counts
1	0.5 ml.	1:500	10.23×10^6
2	0.5 ml.	1:500	10.15×10^6
3	0.2 ml.	1:200	10.27×10^6
4	0.2 ml.	1:200	10.19×10^6

RESULTS

Steady-State Properties of S. cerevisiae at Various Dilution Rates

Figure 11 shows that the population declines linearly as the dilution rate increases and that different concentrations of medium produce essentially the same relationship between population and dilution rate for nitrogen limited media. Also, it is evident from these curves that the point of washout decreases with decreasing medium concentration. These data disagree with the theory in that a theoretical dilution-population curve should have zero slope almost up to the "washout" point, but there is no constant population level with a nitrogen limited culture. The steady-state population versus dilution rate for a glucose limited medium is shown in Figure 12. However, when using the glucose limited medium, the population versus dilution rate curve has a portion that is constant and another portion that falls off almost linearly.

To insure that cell numbers did not reflect anomalies in size of cells, budding phenomena, or cell mass, an investigation of cell dry weight, Kjeldahl nitrogen, and size distributions (electronically) were made at various dilution rates along with Petroff-Hausser counts. Figure 13 illustrates the variation of Kjeldahl nitrogen, cell dry weight, and population with dilution rates for a 20 percent medium concentration. The dry weight curve has a shape that is very similiar to the one for population. However, the Kjeldahl nitrogen was almost constant at the lower dilution rates, but rose to a peak

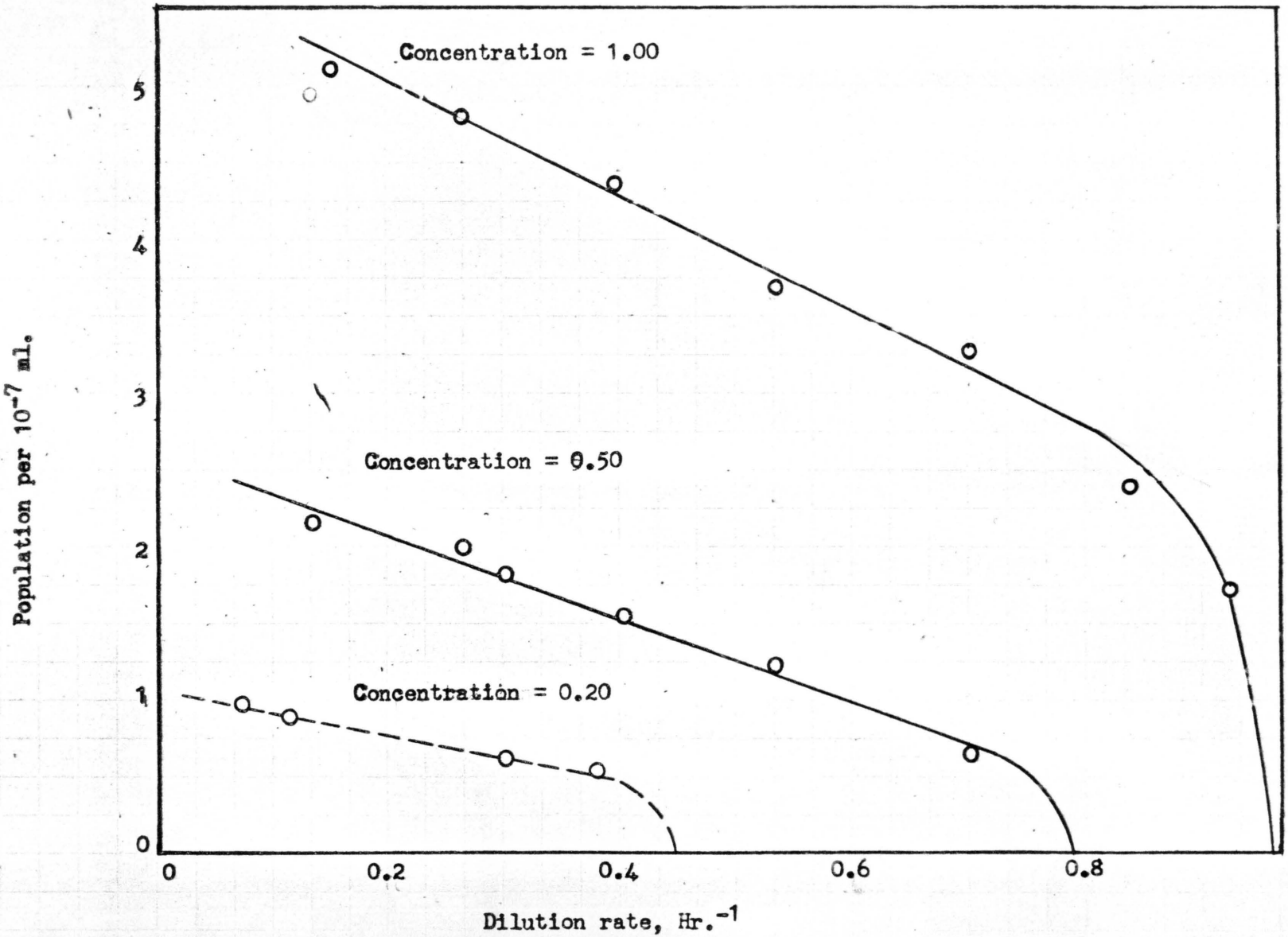


Fig. 11 Change in population with dilution rate for a nitrogen limited yeast culture

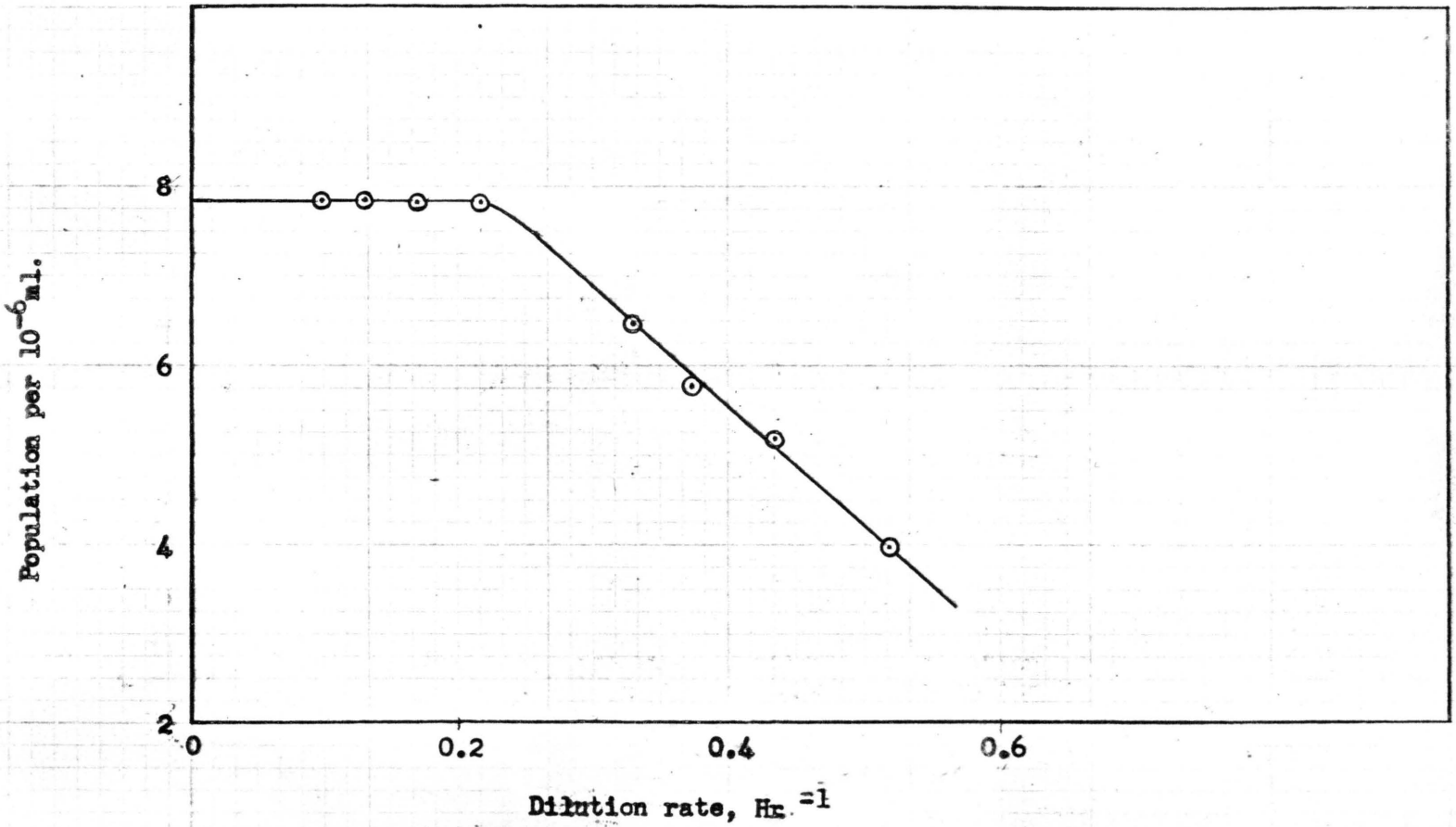
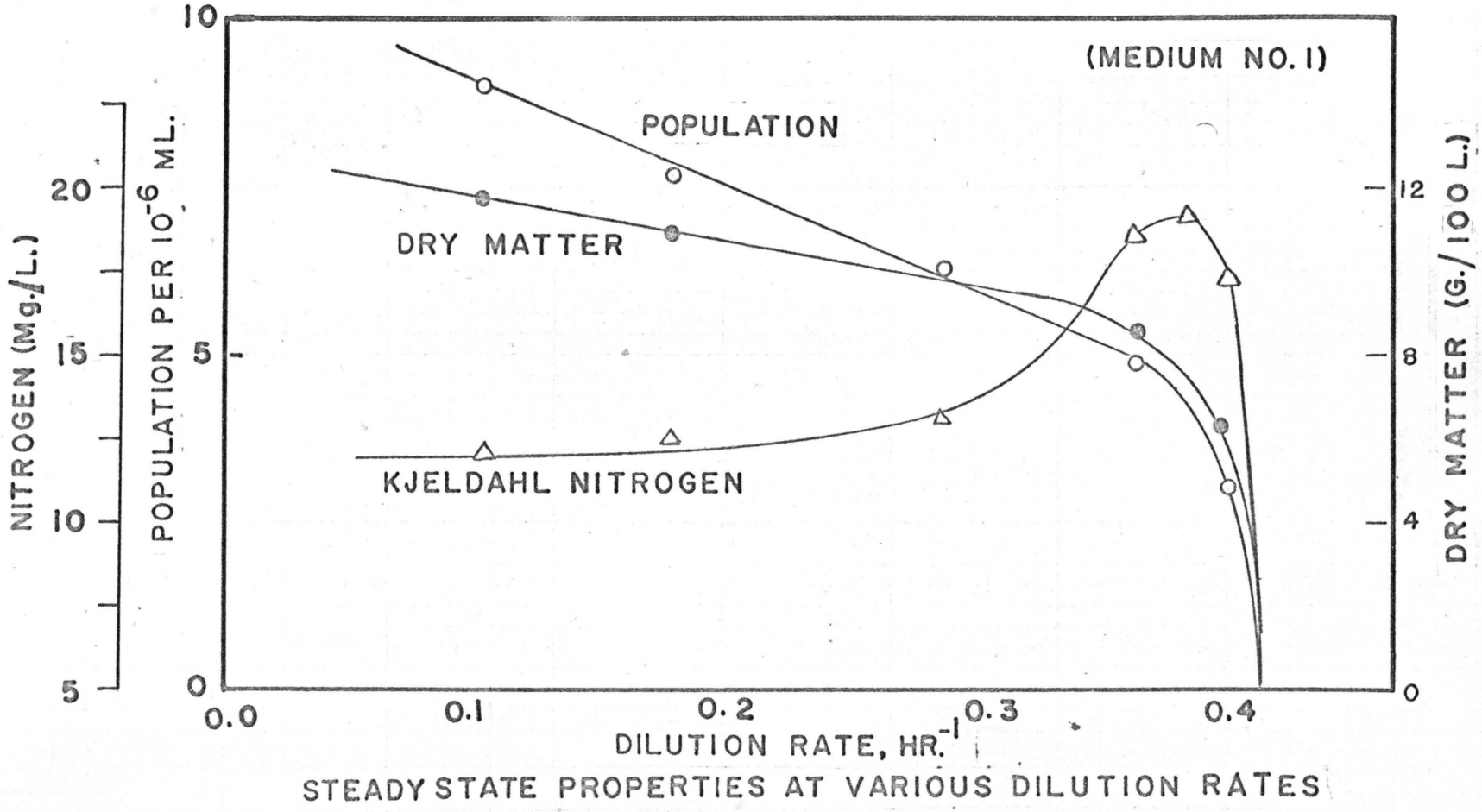


Fig.12 Change in population with dilution rate for a glucose limited yeast culture



STEADY STATE PROPERTIES AT VARIOUS DILUTION RATES

FIGURE 13

at the higher dilution rates. To evaluate the stability of these variables at one steady-state, a series of determinations were made. Table (8) represents the summary of these determinations for one steady-state.

Table 8. Cell Dry Weight and Kjeldahl Nitrogen for the Dilution Rate of 0.20 Over a Period of Time (Medium 1).

Time	Cell Dry Weight (mg/l)	Kjeldahl Nitrogen (mg/l)
0	62.0	12.39
1	59.0	12.50
2	59.0	12.52
3	62.0	12.45
4	61.0	12.50

The fact that the protein nitrogen was increasing as population decreased at the higher dilution rates raised the question of cell size at various dilution rates. Using the Coulter Counter size distributions were obtained for various dilution rates at 20 percent and 40 percent medium concentrations. The average cell size was taken to be the mode of these curves. Figure 14 illustrates the change in average cell size with dilution rate for the two medium concentrations investigated. Therefore, it became evident that the cell size, dry weight per cell, and Kjeldahl nitrogen per cell were definitely increasing as the dilution rate increased. The average dry weight per cell and average Kjeldahl nitrogen per cell were computed by dividing the dry weight per liter and Kjeldahl nitrogen

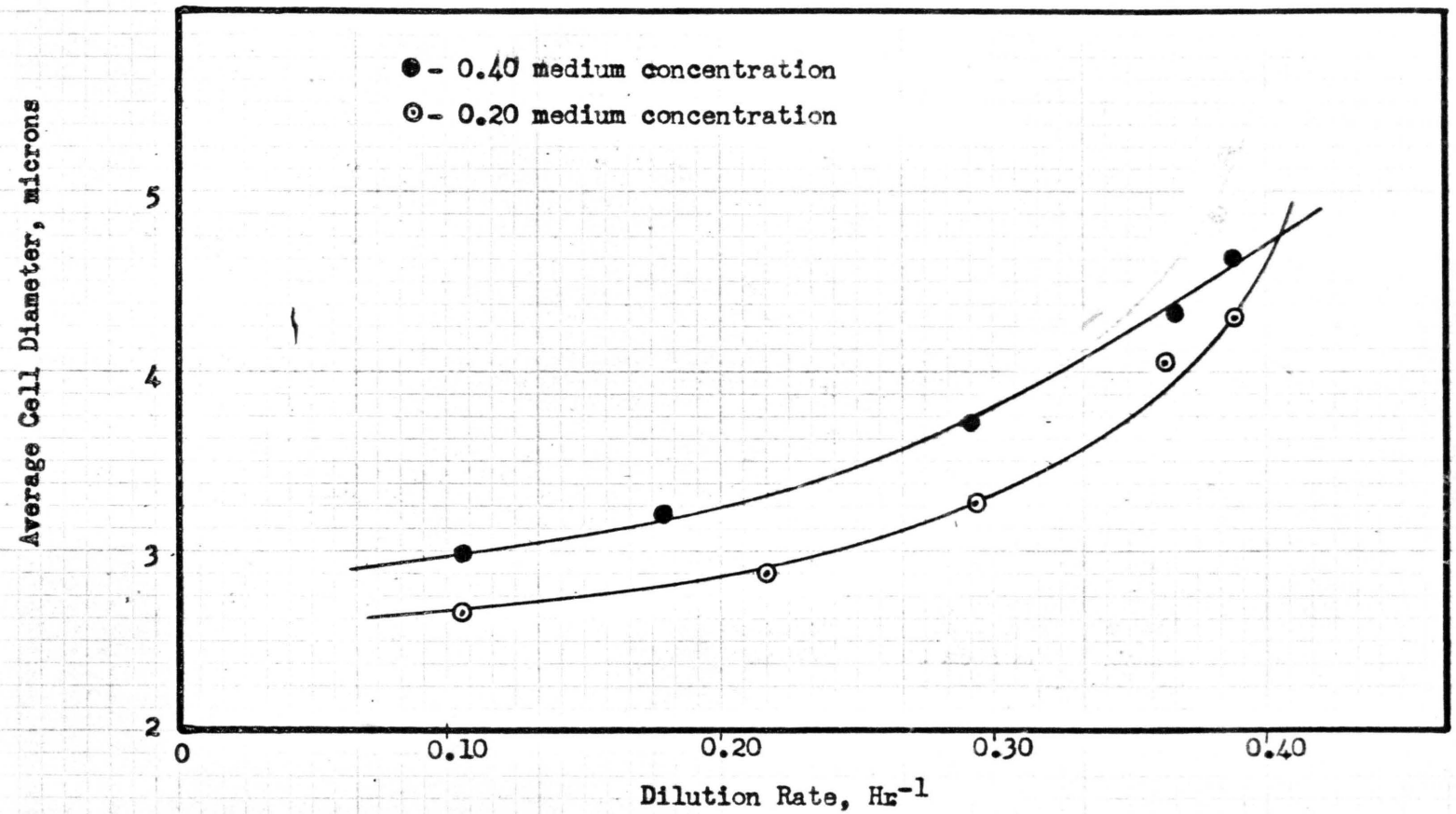


Fig. 14 Change in average cell diameter with dilution rate for different medium concentrations

per liter by the population per liter of effluent. The results of this computation is illustrated in Figure 15 which shows the relationship of the values to dilution rate. An interesting observation from Figure 15 is that the shape of the average cell size curve is very similiar to those for nitrogen and dry weight.

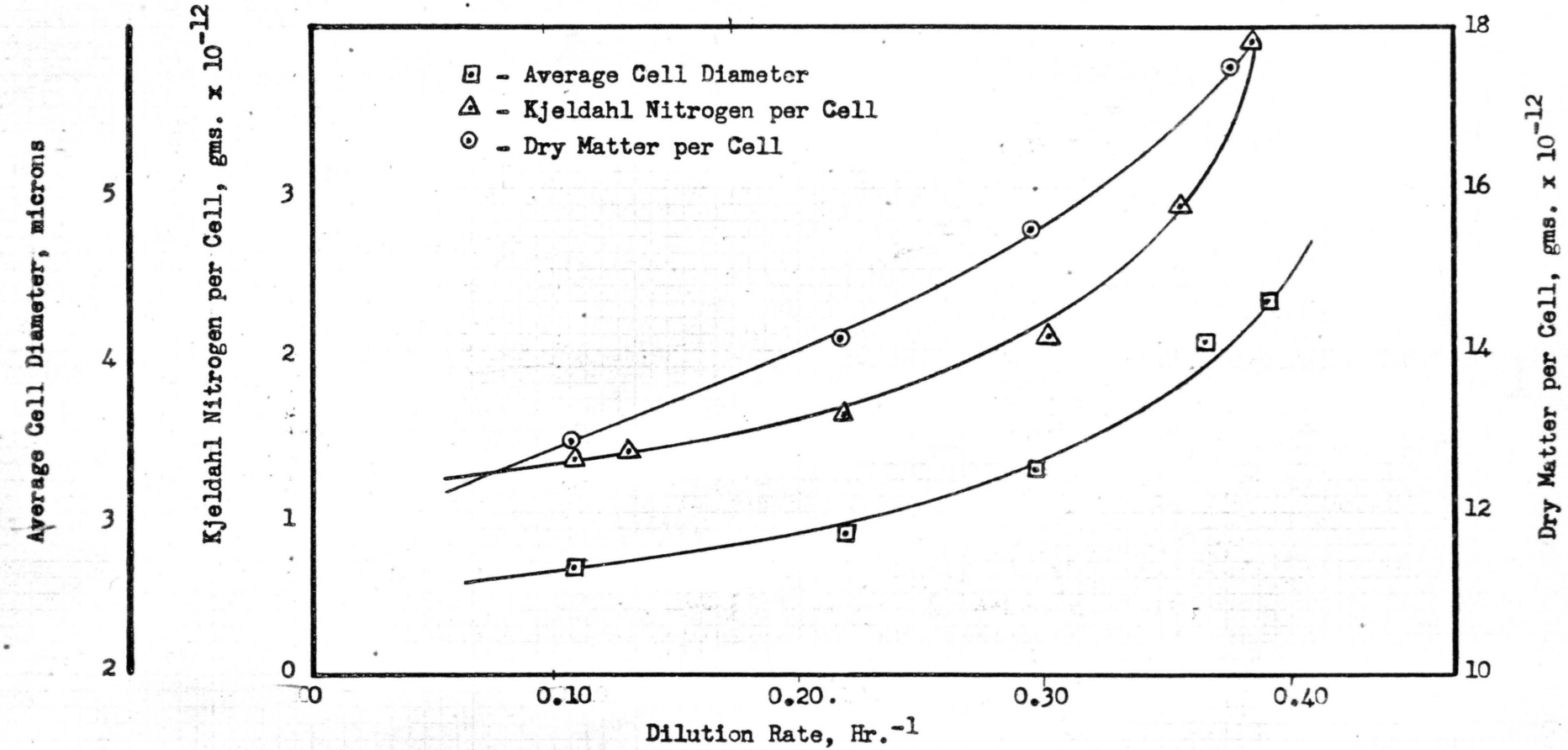


Fig.15 Change in various properties per cell with dilution rate

Response of S. cerevisiae to Step Changes

The work of Shindala (1964) with S. cerevisiae in mixed culture with Proteus vulgaris raised questions concerning the growth rates during transition from one dilution rate to another. It became apparent that the growth dynamics of microorganisms would have to be better understood to properly interpret the data on mixed cultures. Shindala had several occasions where he made an instantaneous increase in the dilution rate and found that the population apparently did not decrease for some time. However, in these experiments electronic counts were taken at one-half hour intervals and no effort was made to follow the population transition continuously. To explore fully the growth rate change during the response to a step change in dilution rate and follow the population as closely as possible until a new steady state was established, it was decided to make a step change in dilution rate. This experiment gave some very unexpected and unusual results. Instead of following a smooth transition from one steady state to another at a constant growth rate (corrected for dilution) the population gave an initial rise in numbers to a peak value, fell below and finally rose to the new steady state. Figure 16 illustrates this phenomena and gives an indication of the stability of the steady states before and after the step change.

The experiment was repeated 10 or 12 times with essentially the same result. Figure 17 is perhaps the best curve obtained and is plotted to illustrate the features of the response. However, when the change in dilution rate was extremely large the population did not exhibit an initial rise in numbers, but a close examination of

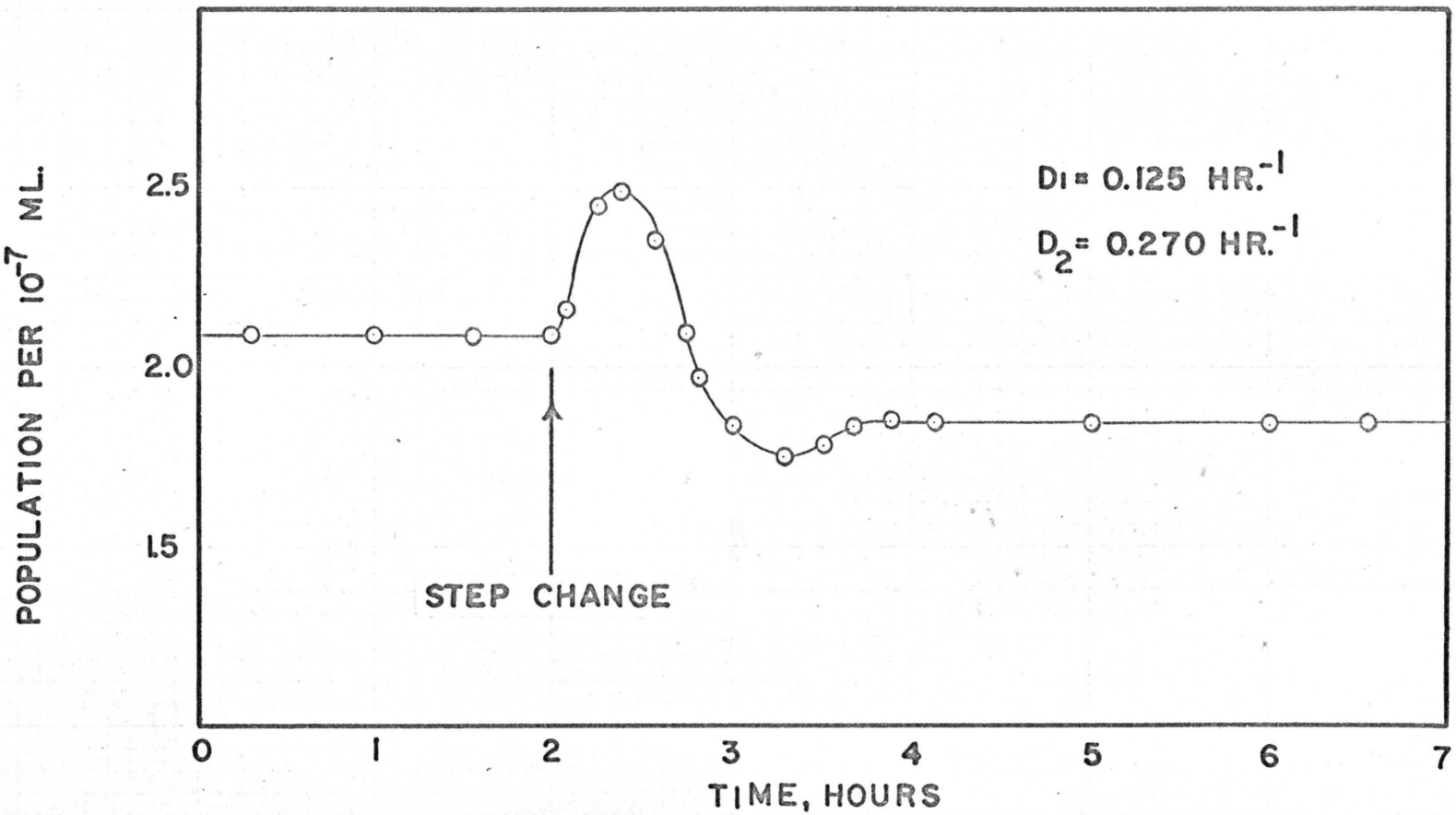


FIG. 16 RESPONSE OF YEAST TO A STEP CHANGE IN DILUTION RATE (MEDIUM 1)

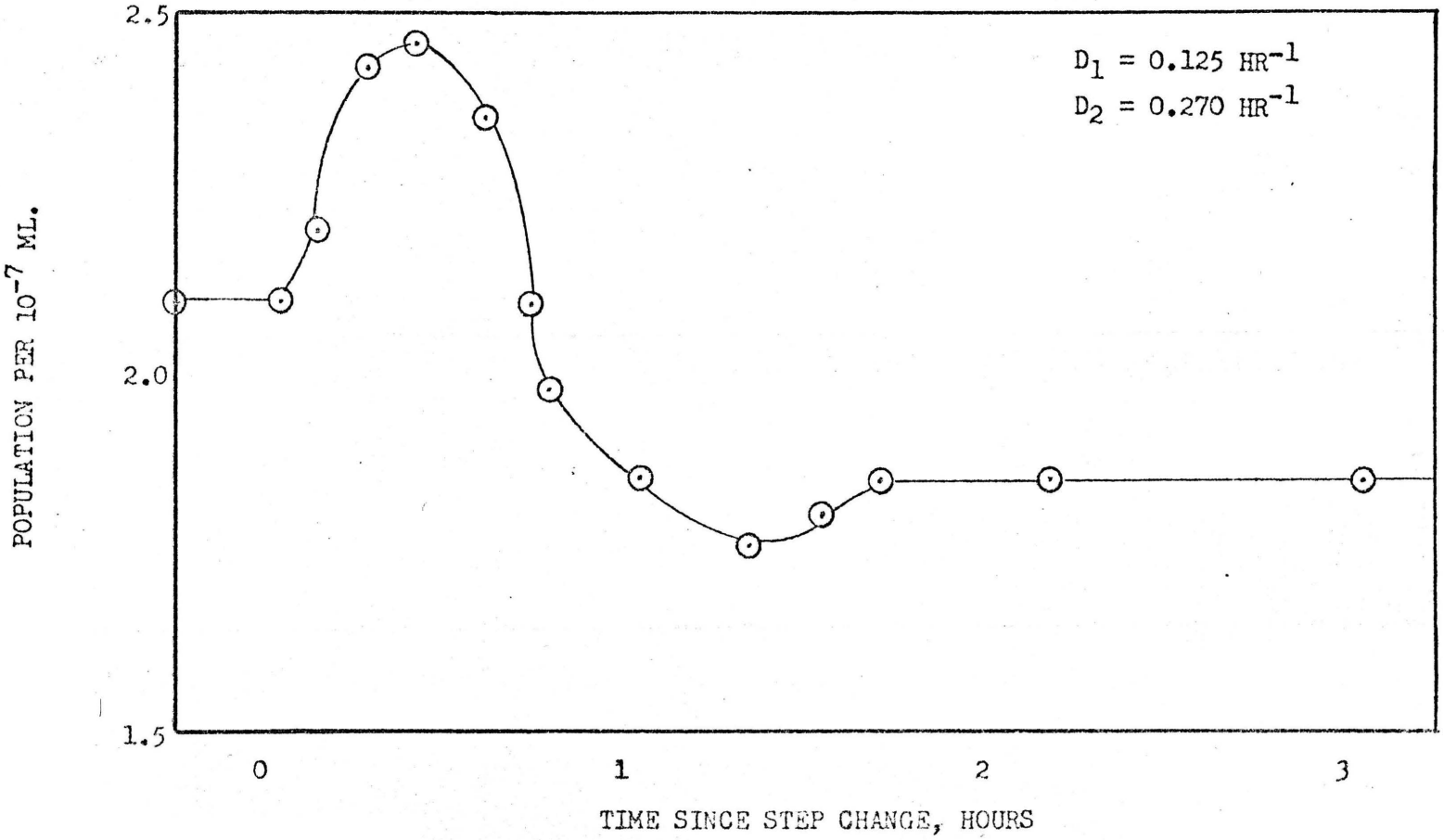


FIG. 17 RESPONSE OF A YEAST CULTURE TO A STEP CHANGE IN DILUTION RATE

the growth rate using equation (3) indicated that growth rate was actually varying a great deal. This type of response is shown in Figure 18. Figures 19 through 22 show typical response to step changes in dilution rates at different medium concentration. The interesting feature in all of these curves is that the populations and thus the growth rates are oscillating in a decaying periodic fashion.

For comparison to nitrogen limited culture a series of step changes in dilution rate for a glucose limited culture was investigated. The response did not oscillate in response to a step change in dilution rate but did exhibit an initial rise in numbers as shown in Figure 23.

The effect of glucose concentration on growth was investigated. It was found that the glucose concentration had no effect on the steady-state populations of the nitrogen limited culture, but when step changes were made in the glucose concentration entering the chemostat an unusual effect was observed. The cell concentration increased until a maximum value was reached after about one hour. Then the population fell below the original steady state and later came back to the initial steady-state population. This phenomena is shown in Figures 24 and 25. Figure 25 also illustrates the specific growth rate as computed from the population curve by use of equation (3). Both the population and growth rate oscillated in a decaying periodic fashion.

The results of a step change in medium concentration are shown in Figure 26. The response is a smooth transition to the new level,

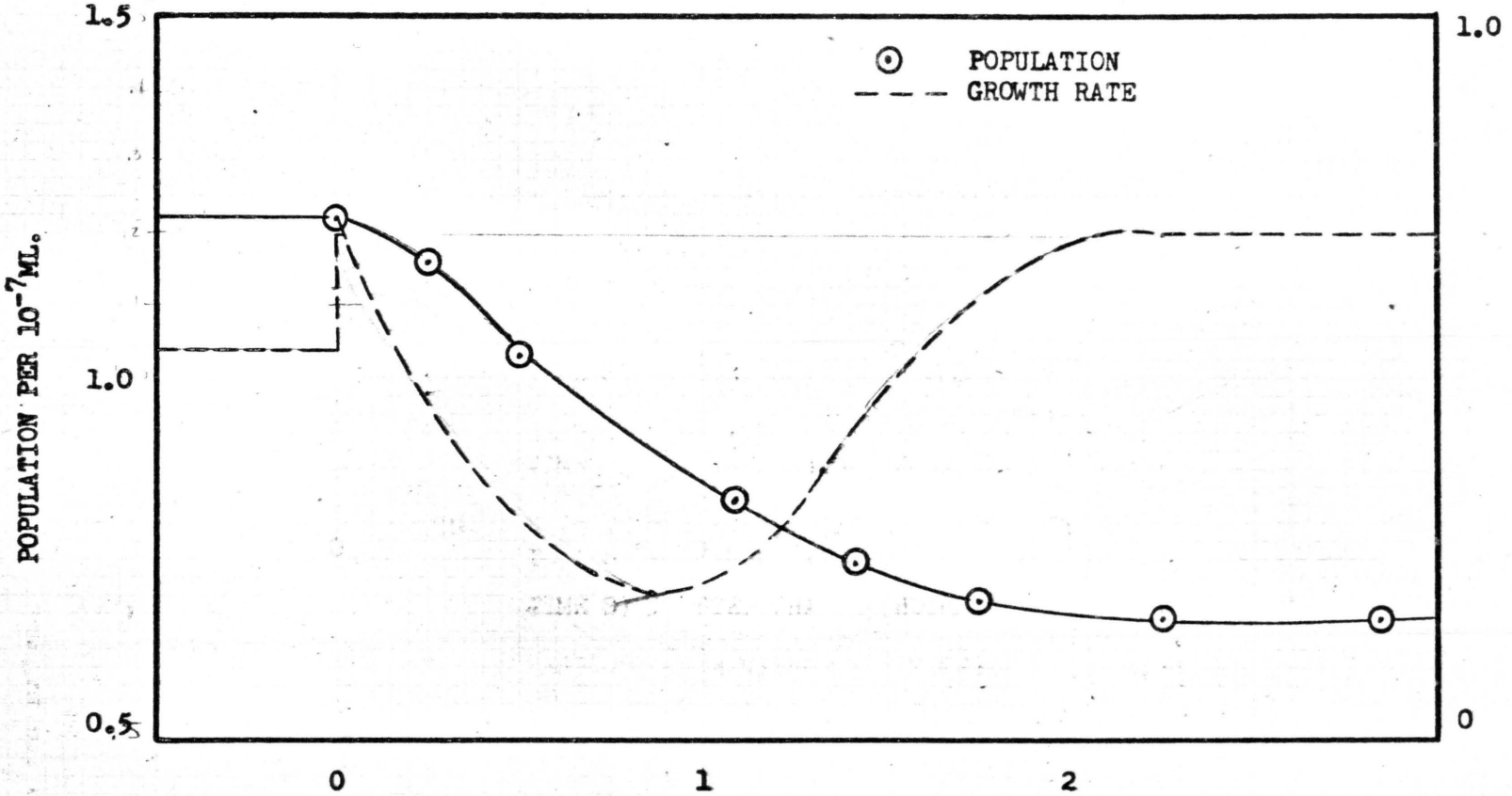


FIG. 18 RESPONSE OF A NITROGEN LIMITED YEAST CULTURE TO A STEP CHANGE IN DILUTION RATE

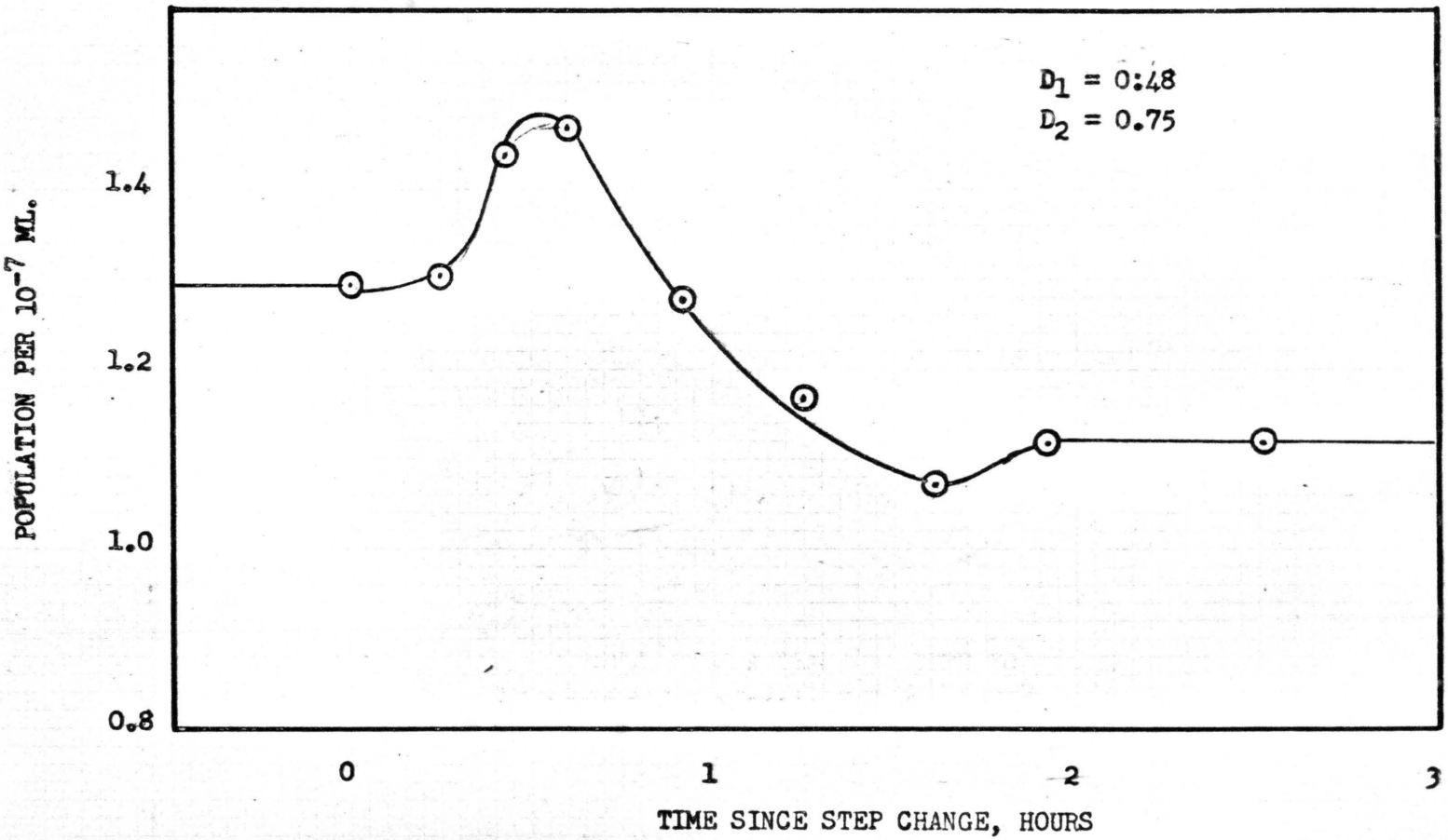


FIG. 19 RESPONSE OF A YEAST CULTURE TO A STEP CHANGE IN DILUTION RATE

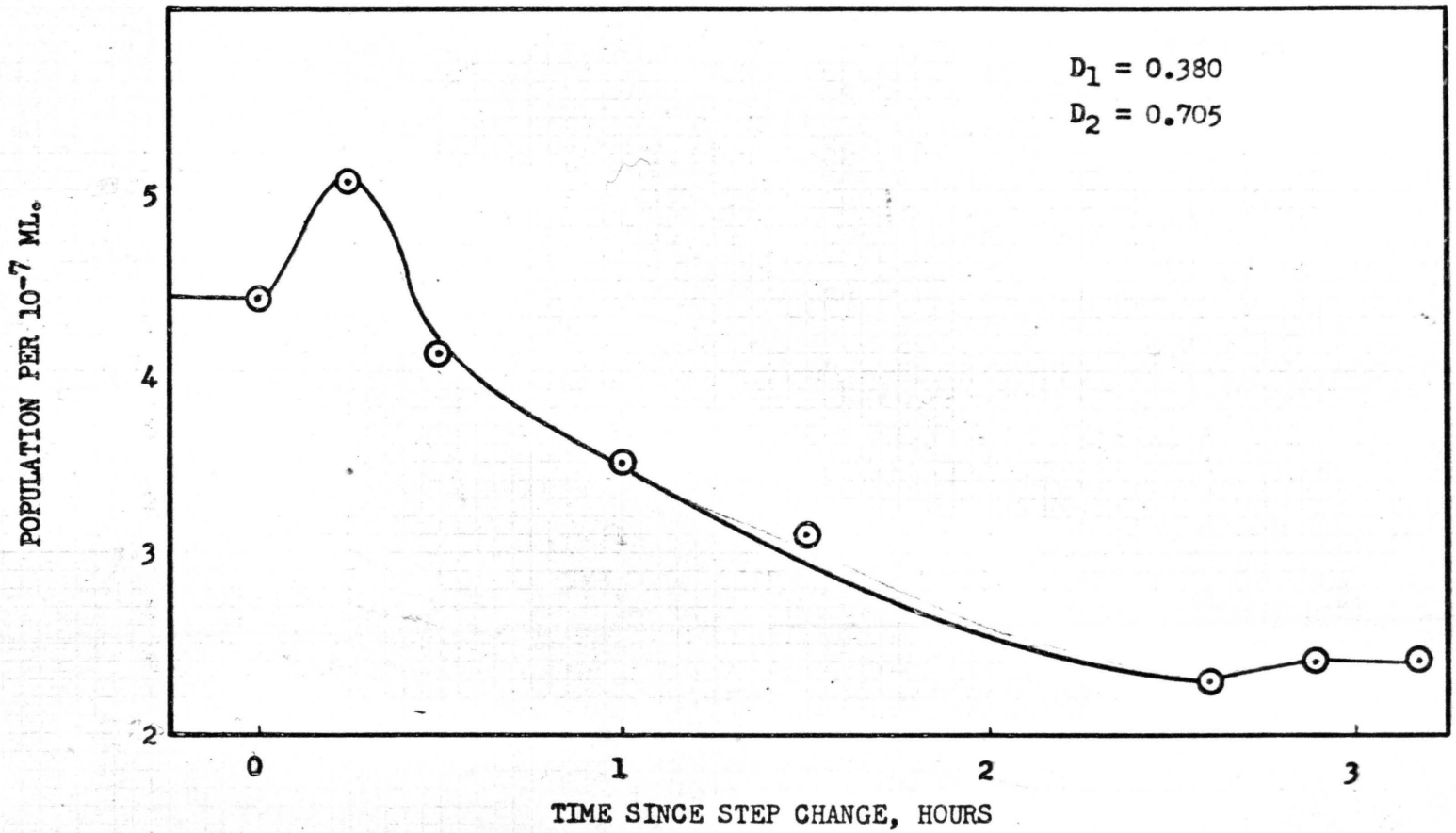


FIG. 20 RESPONSE OF A YEAST CULTURE TO A STEP CHANGE IN DILUTION RATE

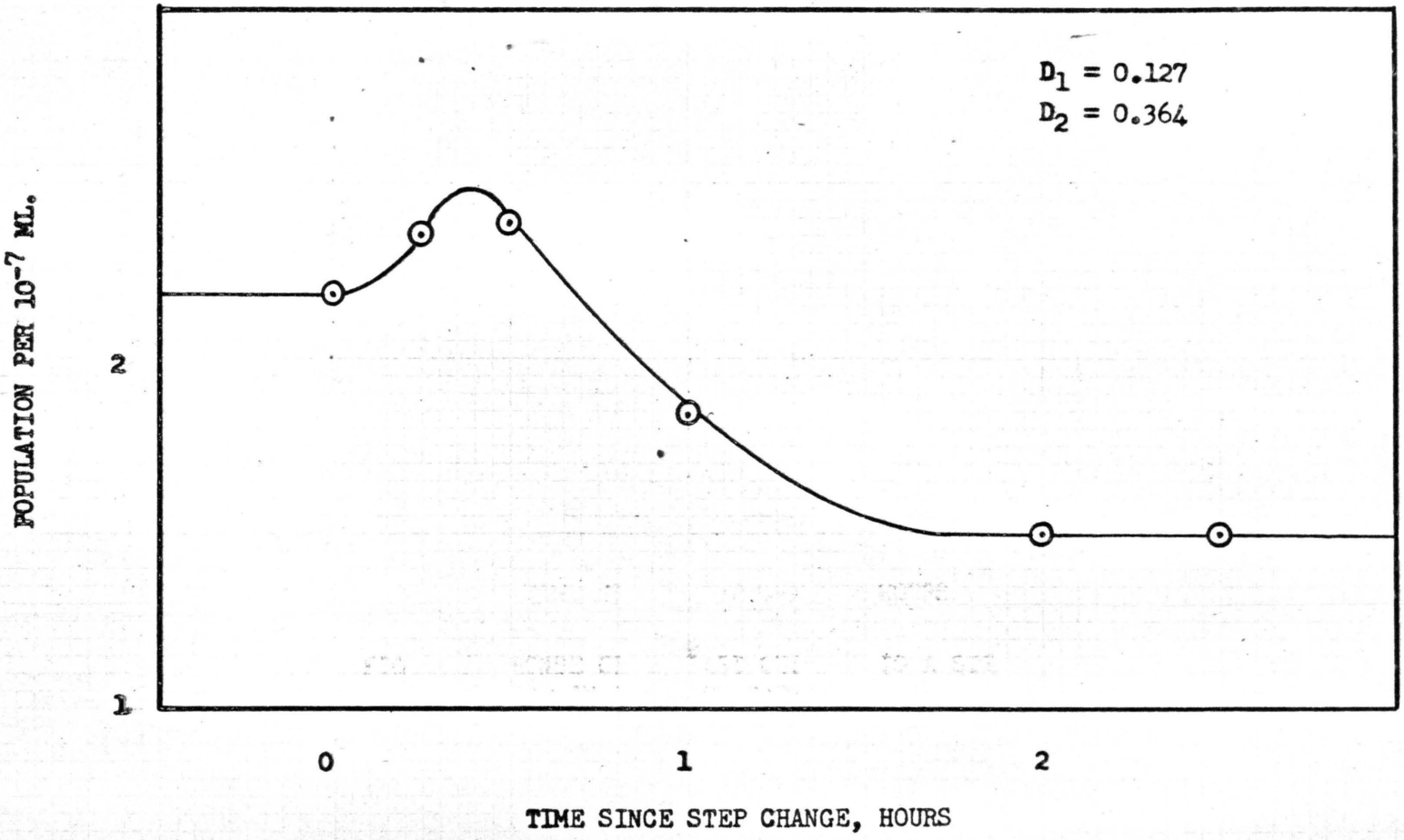


FIG. 21 RESPONSE OF A YEAST CULTURE TO A STEP CHANGE IN DILUTION RATE

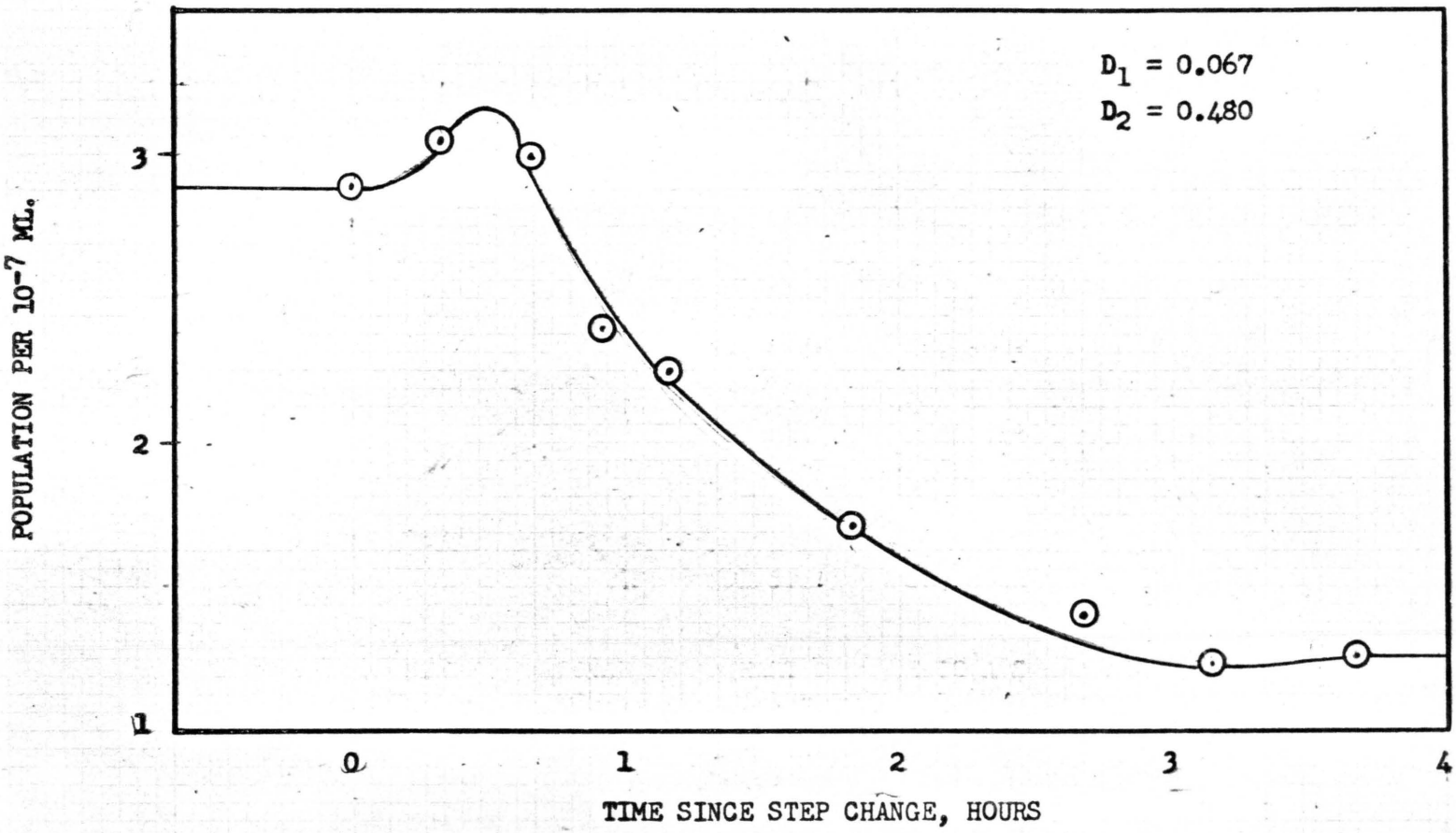


FIG. 22 RESPONSE OF A YEAST CULTURE TO A STEP CHANGE IN DILUTION RATE

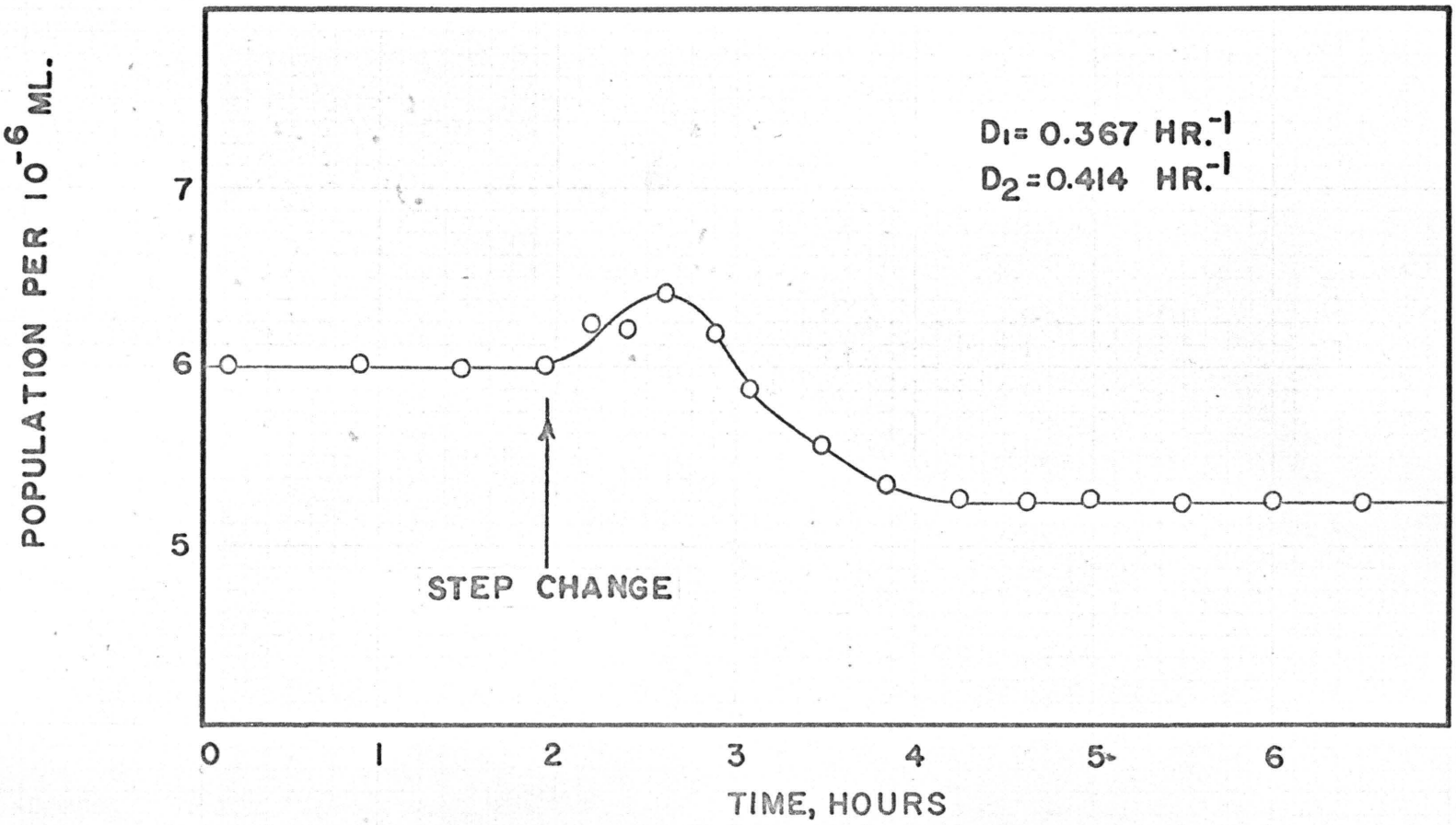


FIG. 23 RESPONSE OF YEAST TO STEP CHANGE IN DILUTION (MEDIUM 2)

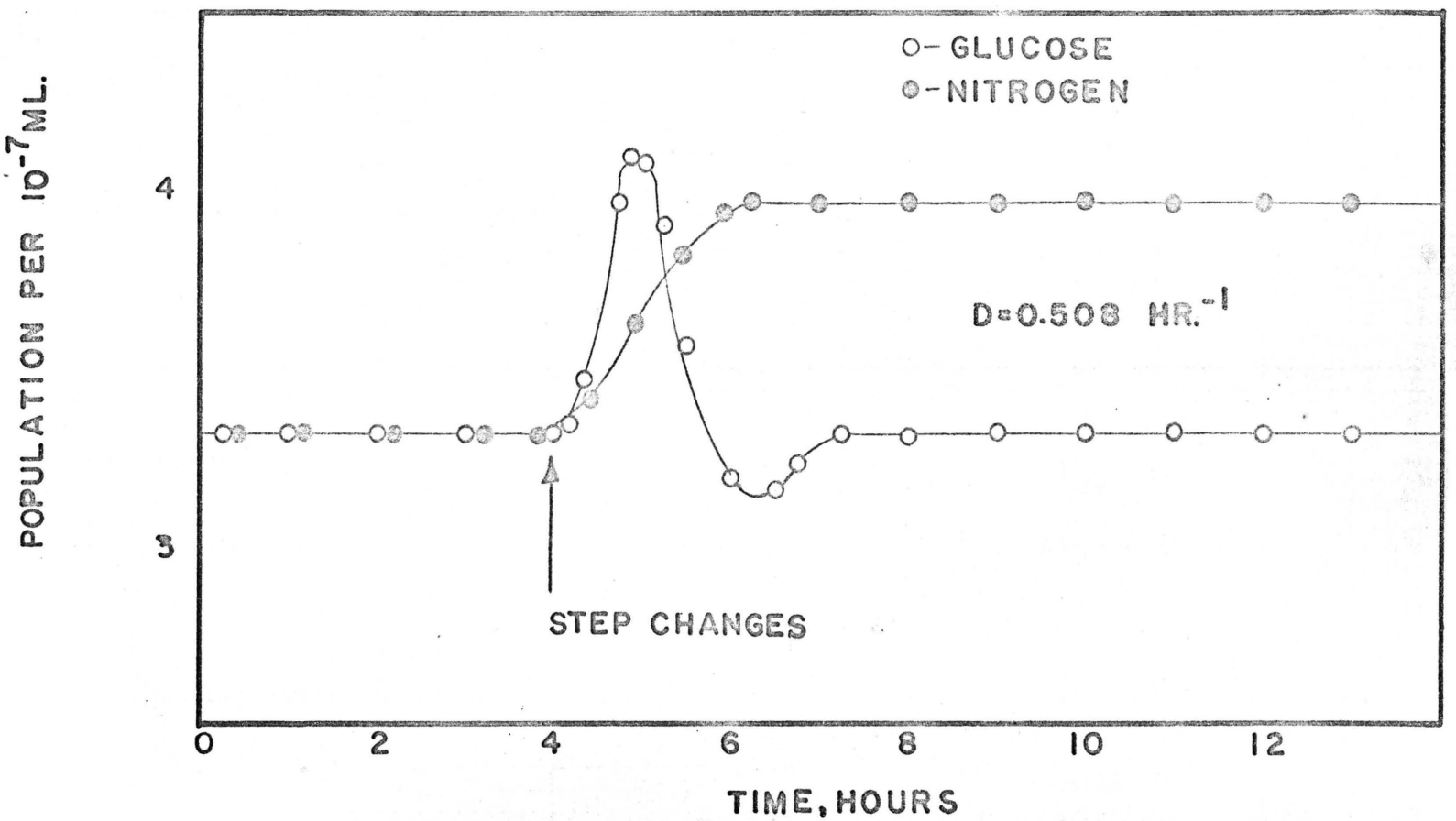


FIG. 24 RESPONSE OF YEAST TO STEP CHANGES IN GLUCOSE AND NITROGEN (MEDIUM 1)

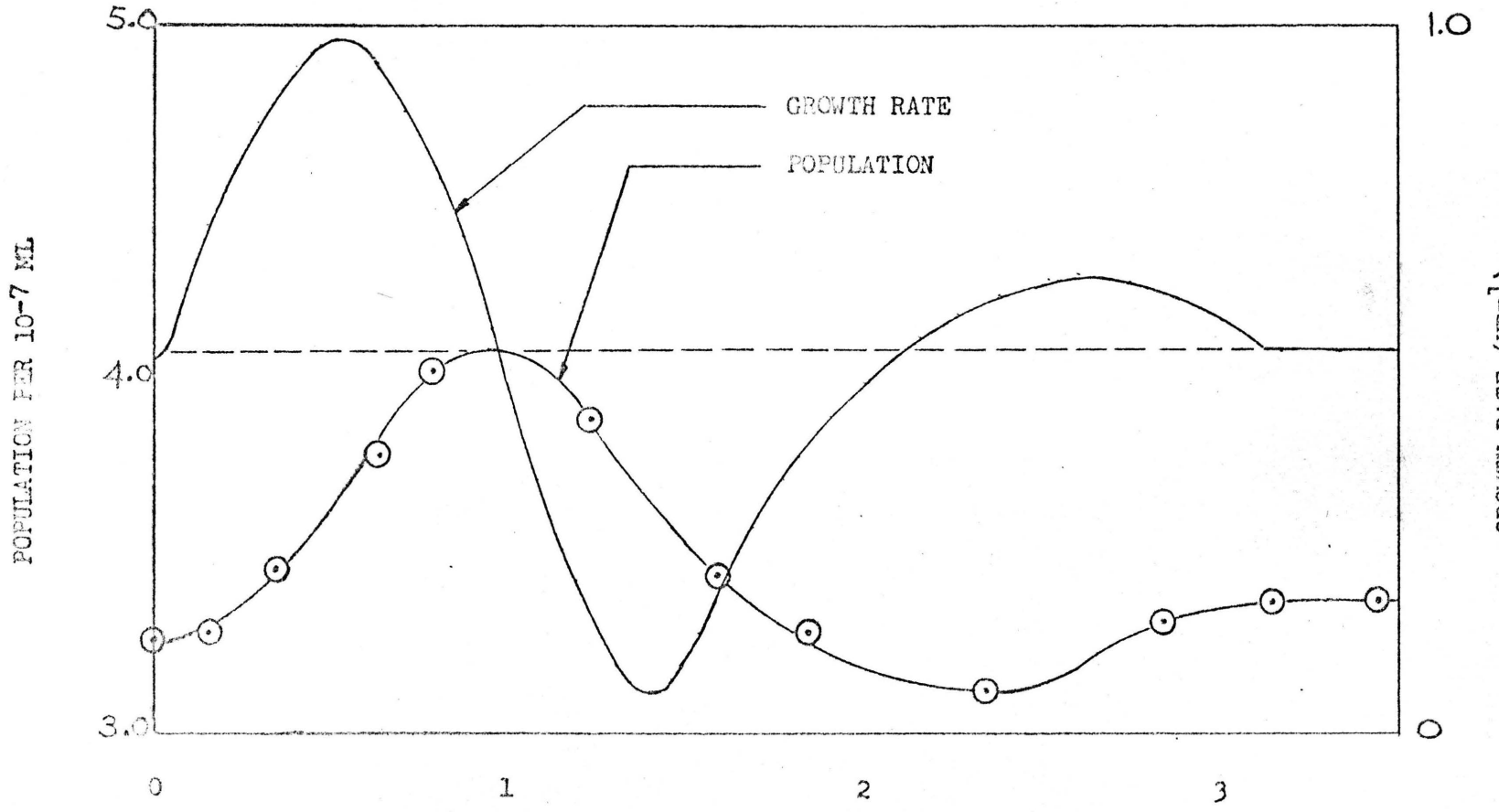


FIG. 25 RESPONSE OF A NITROGEN LIMITED YEAST CULTURE TO A STEP CHANGE IN GLUCOSE CONCENTRATION

which normally indicates a first-order system. Also, the response to a step change in nitrogen (nitrogen limited culture) was investigated and a response similiar to that found for medium concentration was observed. Figure 27 shows the response of a step change in nitrogen source for a nitrogen limited yeast culture.

Figure 28 illustrates the effect of stopping the continuous media flow for 0.33 hour then returning to the original rate of flow. This could be termed a pulse change in dilution rate. During the time in which the dilution rate was zero the yeast grew in batch culture and attained the higher population shown. Once the flow was started again, the population decreased to the original steady-state population, but not at a constant growth rate (corrected for dilution). Instead, the population varied in a drifting oscillatory fashion and thus the growth rate also varied in an oscillatory manner. Figure 29 illustrates another typical pulse in dilution rate but with the scale for population expanded to show the oscillation more clearly.

All of the oscillations reported here were of population as it was assumed that population gave an accurate reflection of cell mass. However, since this was an assumption, it became necessary to investigate other variables during the oscillations. Because of the large samples required of chemical assays of Kjeldahl nitrogen and dry weight these could not be recorded continuously. To investigate these variables steady states were established, then a step change

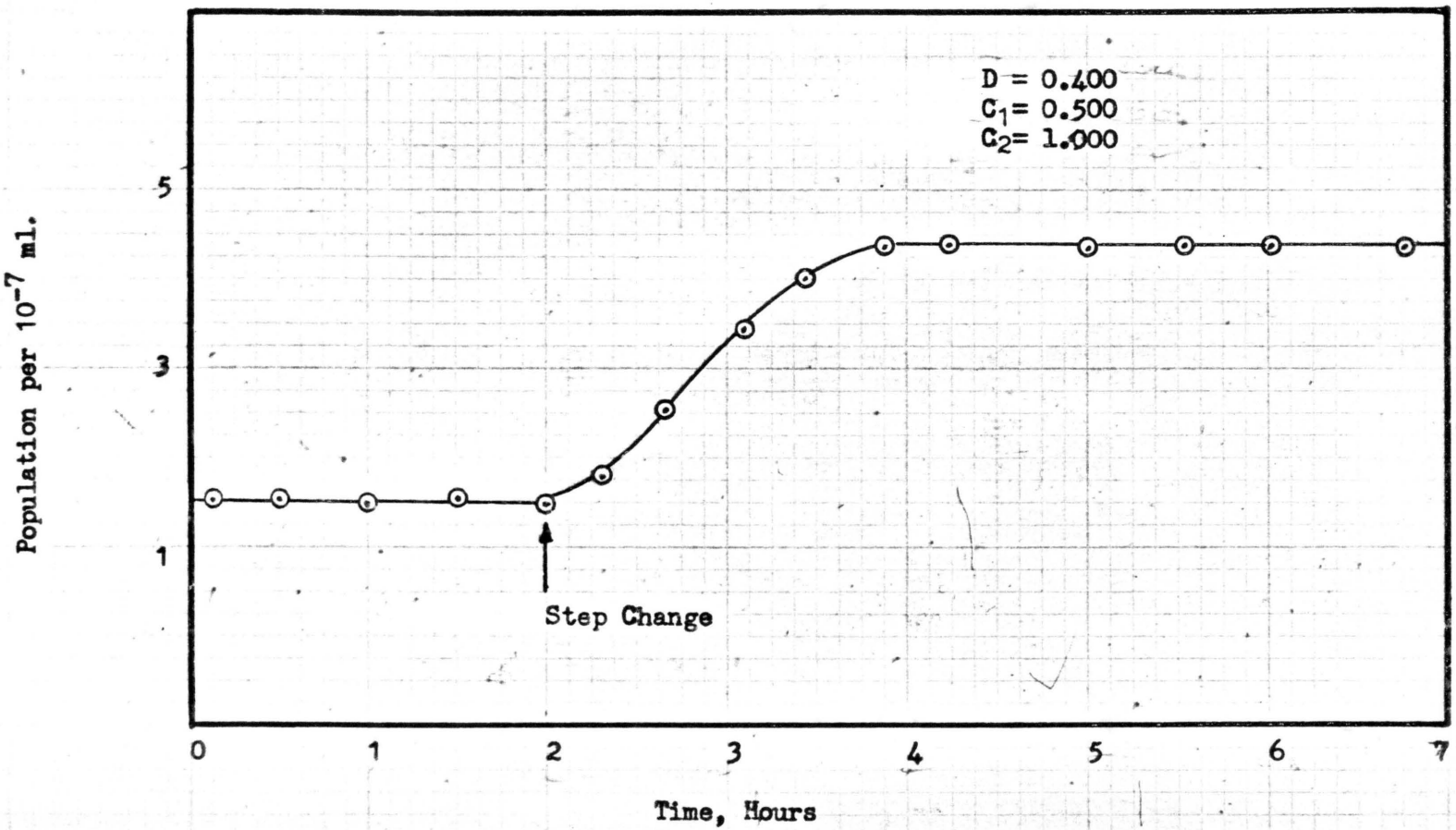


Fig. 26 Response of a nitrogen limited yeast culture to a step change in medium concentration.

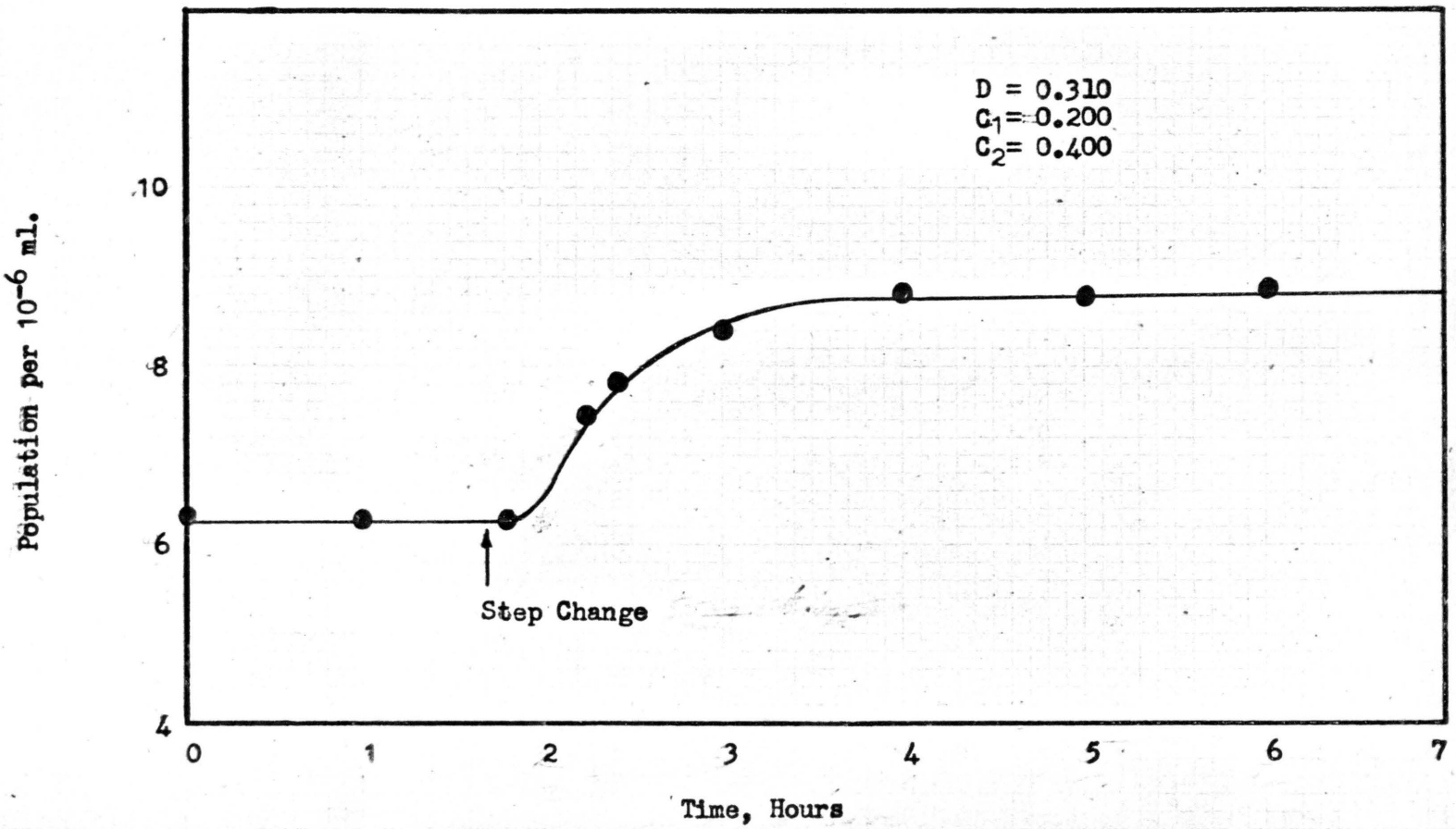


Fig. 27 Response of a nitrogen limited yeast culture to a step change in nitrogen concentration

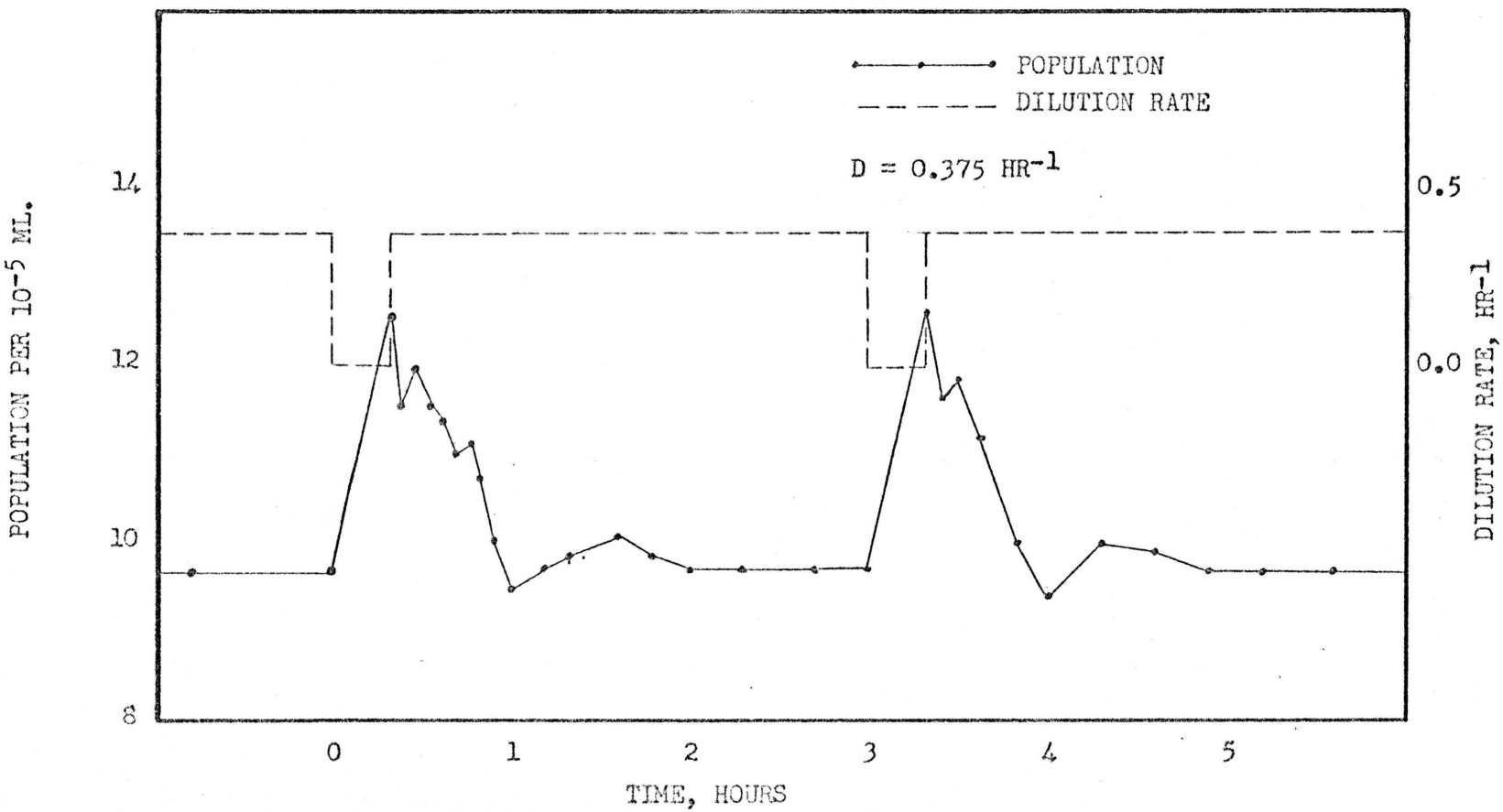


FIG. 28 RESPONSE OF A NITROGEN LIMITED YEAST CULTURE TO A SQUARE WAVE CHANGE IN DILUTION RATE

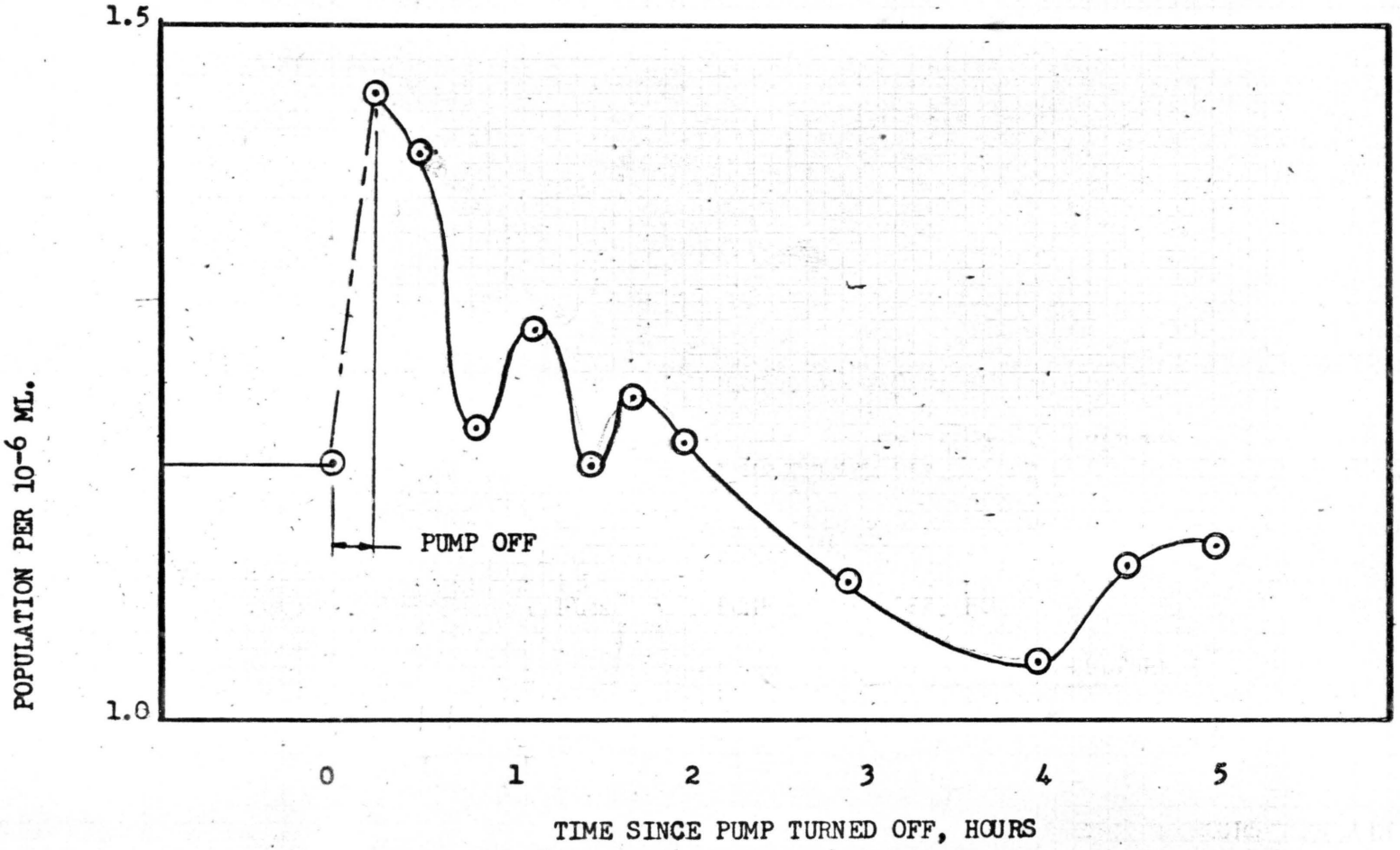


FIG.29 RESPONSE OF NITROGEN LIMITED YEAST CULTURE TO SQUARE WAVE CHANGE IN DILUTION RATE

was made in dilution rate. Near the peak of yeast oscillation, samples were taken to measure cell dry weight, Kjeldahl nitrogen, and Petroff-Hausser counts. Table (9) shows that, near the peak of oscillation, Coulter Counter counts, chamber counts, dry weight, and Kjeldahl nitrogen are greater in approximately the same proportion as at the original steady state.

Table 9. Correlations Near Peak After Dilution Rate Change*

Time	Population	Dry Weight	Kjeldahl N.
During steady state	6.62 per 10^{-6} ml.	0.096 gm/liter	13.0 mg/liter
40 min. after change	7.41 per 10^{-6} ml.	0.101 gm/liter	15.5 mg/liter

*Initial D = 0.252, new D = 0.400, one-fifth medium concentration

Sinusoidal Forcing of S. cerevisiae in Ammonium Sulfate Limited Medium

The response to dilution rate and glucose concentration were found to be decaying periodic oscillations, and since such complicated responses are not amenable to the usual systems analysis techniques, it was decided to change from step changes to sinusoidal forcing. By use of the sinusoidal forcing the time constants can be obtained for a given system graphically by use of the Bode diagram.

Two types of sinusoidal forcing were utilized, both using the sinusoidal flow generator previously described. First, it was decided to force the flow rate sinusoidally and then to force the concentrations of certain chemicals sinusoidally. Also, some preliminary investigation on the response of the yeast to sine forcing of pH in the culture vessel was accomplished, but the results were not satisfactory.

The time constants for response to change in dilution rate were obtained in two separate experiments using input waves of two different amplitudes at various frequencies. First, medium was pumped at a constant base rate with a slow pump while varying a faster pump sinusoidally. This allowed some flow at all times thus making sampling easy. The faster pump was caused to pump sinusoidally at different frequencies using different speed motors and different gear arrangements on the interval timer flow generator. Each frequency was allowed to continue over eight or ten sine wave periods before counting was begun, then the population was followed electronically for two or three periods to obtain enough points to describe the outflow curve. Figure 30 illustrates a typical outflow curve for a sinusoidally forced

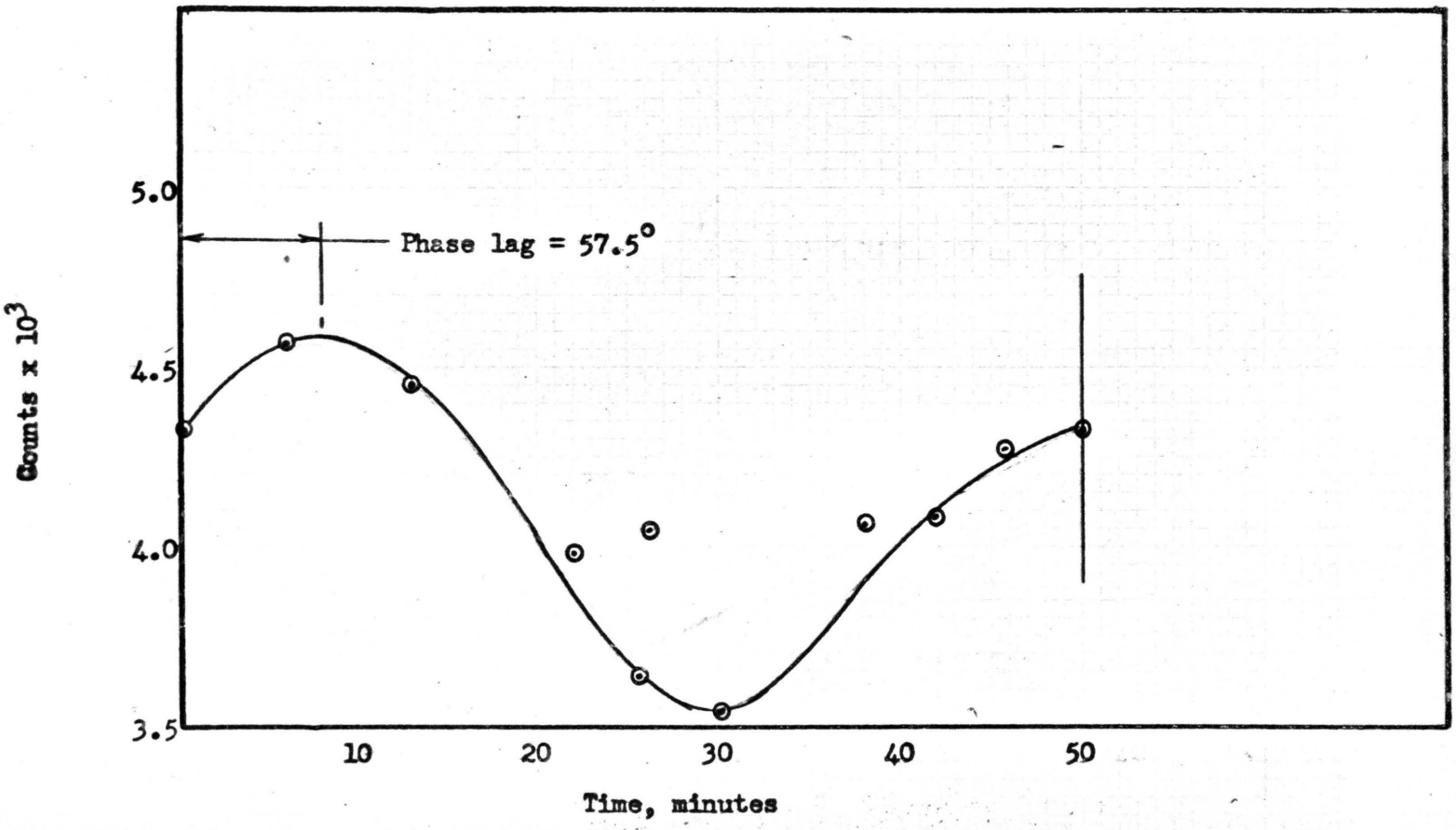


Fig. 30 Typical response of Yeast to sinusoidal variation of dilution rate

continuous culture of yeast. For the initial study of dilution rate, the average dilution rate was 0.375 hr^{-1} with an amplitude of 0.206 hr^{-1}. Several of these outflow curves were obtained over a range of frequencies with the phase shift and magnitude ratio being computed as described previously. These values were then plotted on a Bode Diagram shown in Figure 31. From this Bode plot the system was found to be a third-order system (a 270° phase shift indicated a third-order system) which consisted of a second-order quadratic system followed by a simple first-order system. The time constant for the first-order system was determined as described in the systems analysis section, and the time constants for the second-order system were determined using the template shown in Figure 8. The experimentally obtained data did not fit the template perfectly, but was sufficient to obtain the critical frequency.

As a check on the first Bode Diagram for dilution rate we repeated the experiment using different average dilution rate and amplitude. This time the base dilution rate used was 0.250 hr^{-1} with an amplitude of 0.125 hr^{-1}. Figure 32 shows the Bode plot for this experiment which is very similiar to the original diagram for dilution rate. Also, the time constants obtained were very close to the original ones, and are shown in Table (10), which summarizes the time constants determined in this research.

Next, the time constants for response to glucose medium and nitrogen concentrations were investigated using frequency response analysis. The sine waves were generated using the sine flow generator described by Sutherland and Bungay (1965) with some modifications. An

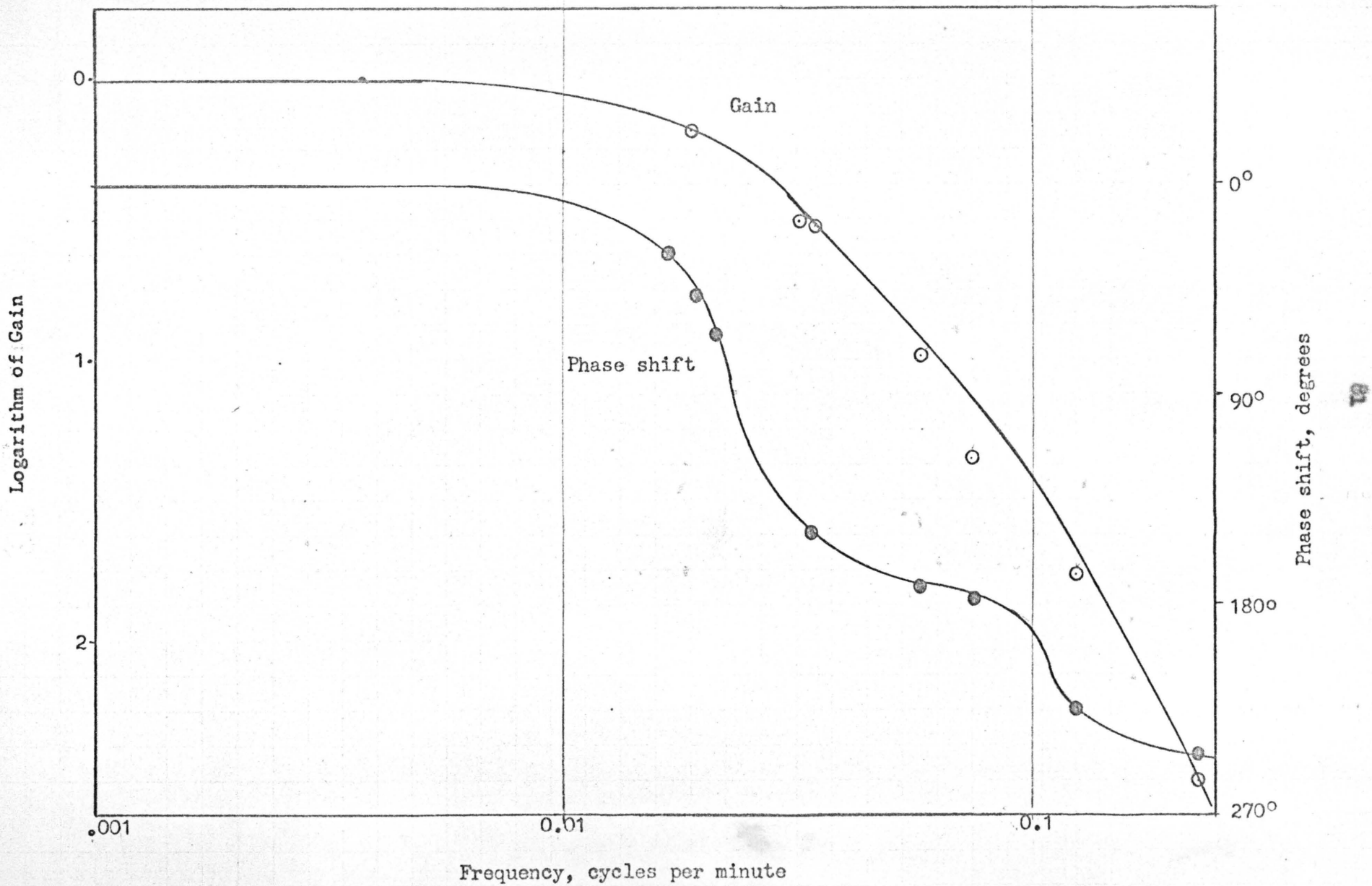


Fig. 31 Bode diagram for yeast growth versus dilution rate

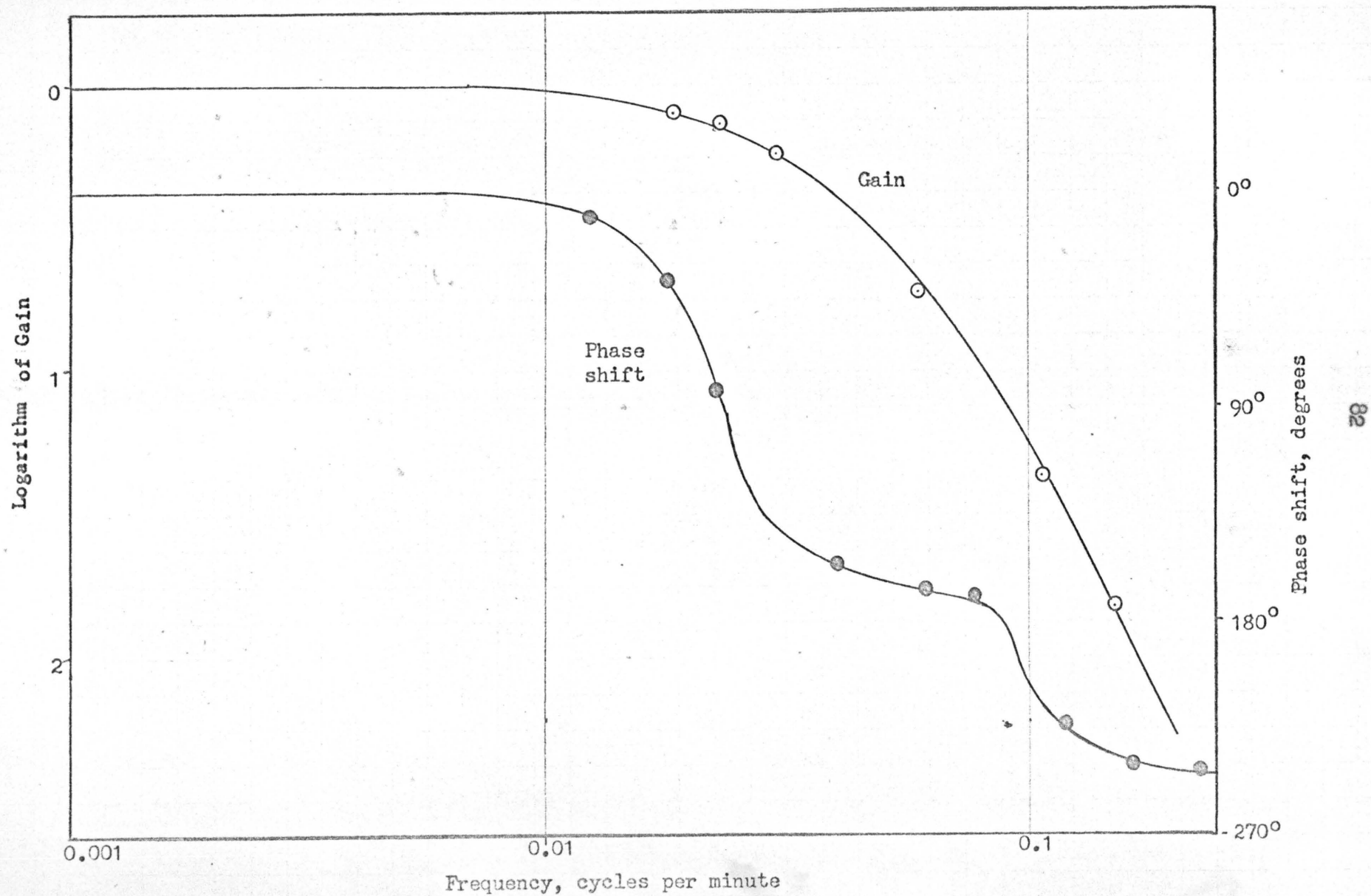


Fig. 32 Bode diagram for growth versus dilution rate

additional mercury switch was added beside the existing switch, but in reverse position. This allowed two pumps to be connected to the generator switches and thus one pump would be pumping while the other would be off. Using matched pumps two different concentrations of substrate could be pumped in a fashion which would allow a sinusoidal variation of concentration while the dilution rate remained constant. The procedures of establishing outflow waves were the same as previously described for the dilution rates experiment.

Figure 33 gives a typical response to sinusoidal forcing of medium concentration, and Figure 34 is the Bode plot for response to medium concentration. The time constants for this system are listed in Table (10).

Preliminary experiments were unable to produce sine waves in response to forcing of the glucose concentration. Thus the critical frequency may be out of the range of our generator. Also, the response to sine forcing of nitrogen concentration was explored and a large quantity of data accumulated, but these data seems to be too highly complicated for proper interpretation at this time. Also, some experiments with sinusoidal forcing of dilution rate did not produce acceptable output sine waves.

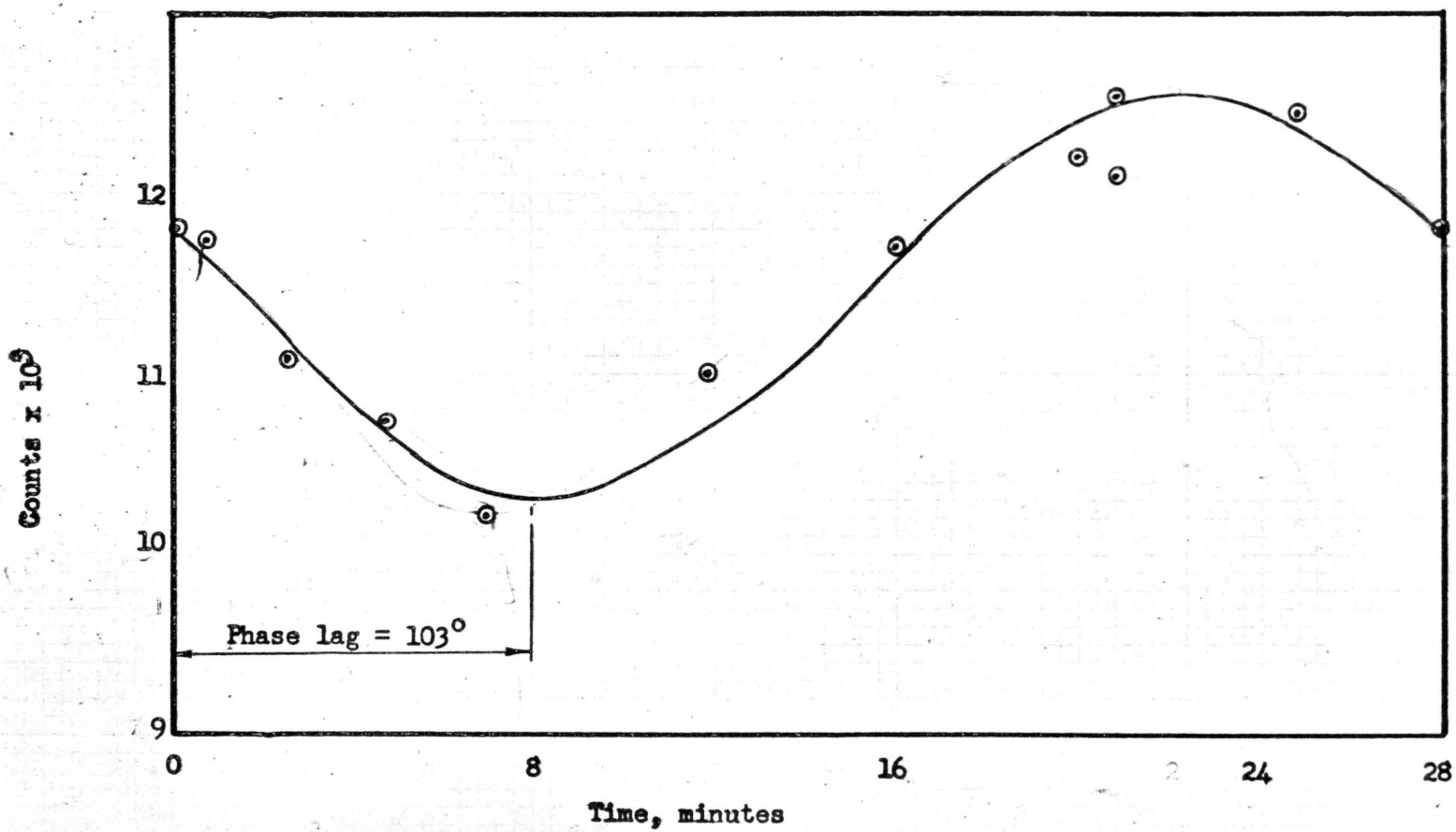


Fig. 33 Typical response of Yeast to sinusoidal variation of medium concentration

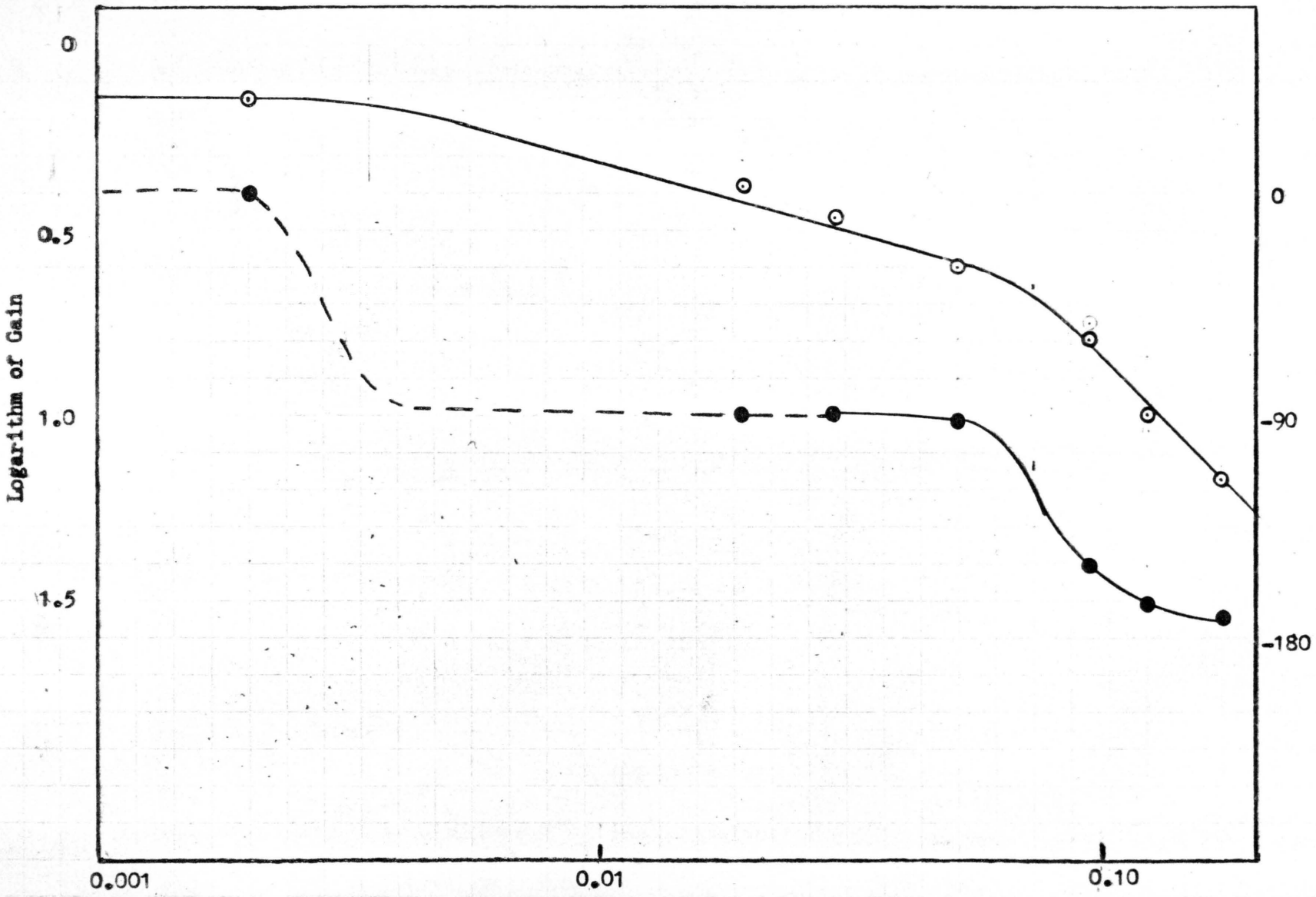


Figure 34 Bode diagram for growth versus medium concentration

Table 10. Summary of Time Constants for Response of S. cerevisiae to Dilution Rate and Medium Concentration

Number	Variable	Time Constants
1	Dilution Rate	8.33 and 43.2 Minutes
2	Dilution Rate	8.25 and 38.7 Minutes
3	Medium Concentration	10.22 Minutes

Effect of E. coli on the Growth of S. cerevisiae

Shindala (1964) investigated the effect of contaminating a growing culture of S. cerevisiae with E. coli. In this investigation S. cerevisiae was allowed to attain a steady-state population in a continuous culture vessel and then inoculated with a growing culture of E. coli. Samples were collected at half hour intervals until the concentration of the yeast became too low to count.

The theory of contamination by Golle (1953) predicts that the con tamination of a slow growing organism with a fast growing organism in a continuous flow system would result in the washing out of the slower growing organism. S. cerevisiae is a much slower growing organism than the coliform and was washed out of the system; E. coli increased in number until it reached a steady-state condition. Figure 35 (taken from Shindala's Ph. D. thesis) shows this contamination. When Shindala's data was treated by equation (3) it was noted that the yeast declines rapidly once the population of E. coli becomes large and finally the yeast growth rate goes to zero. This growth rate is shown in Figure 35 and it is interesting to note that while the growth of the yeast has stopped the growth of E. coli is increasing rapidly.

The decline of the growth rate of S. cerevisiae while substrate was still plentiful raised questions about the factor involved in this contamination phenomena. Was the contamination due to competition for the limiting substrate, inhibitation, or some other factor? To investigate these possibilities a two-stage continuous culture system previously described was built. This allowed pumping of a continuous constant cell

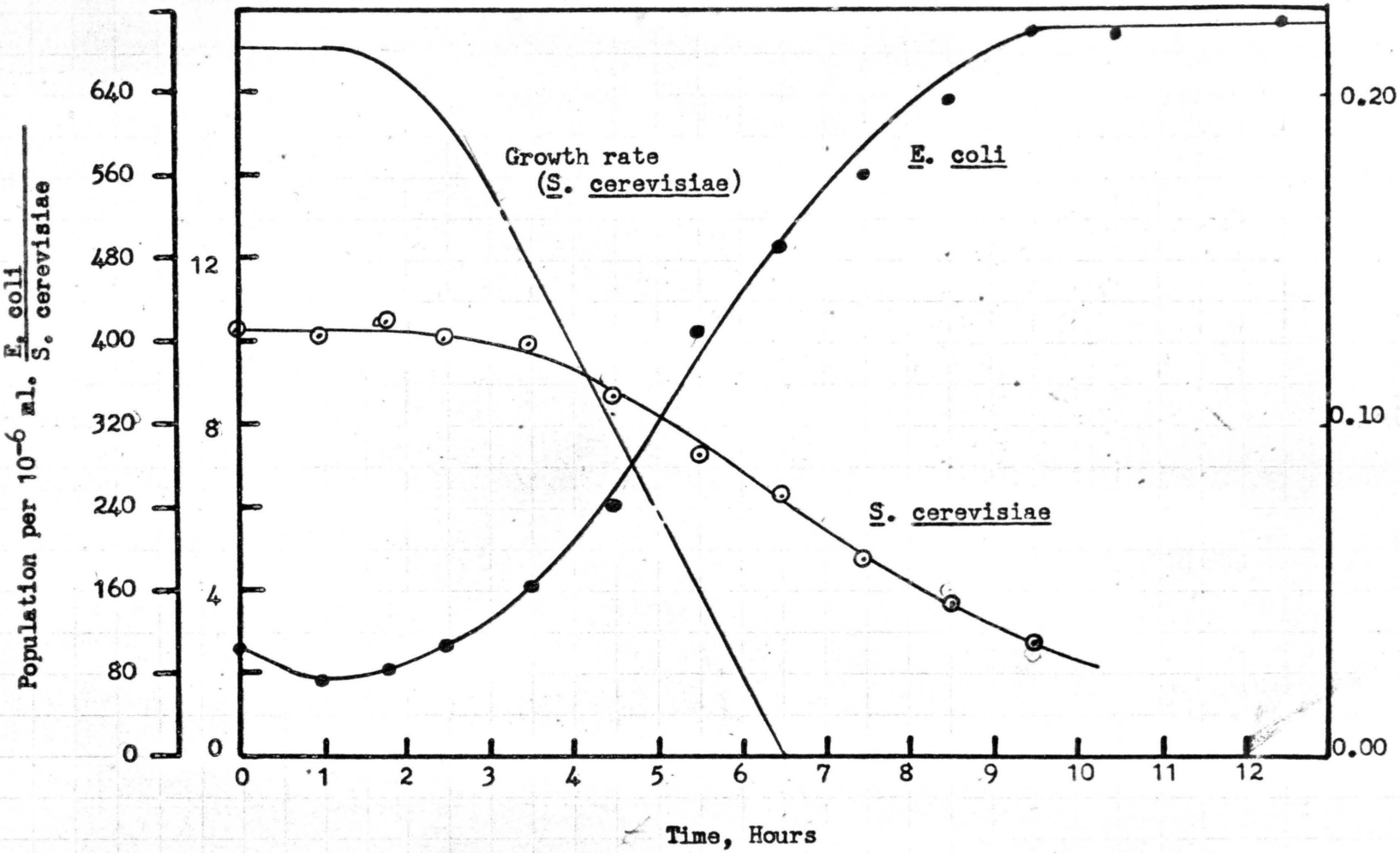


Fig. 35 Contamination of S. cerevisiae with E. coli

concentration from Stage I into the mixed culture of Stage II which provided time to study the interaction. After being charged with medium both vessels were inoculated, the first with S. cerevisiae and the second with E. coli, and allowed to grow in batch culture until turbidity was obvious. Then the continuous flow of medium through the system was begun and the populations allowed to reach steady states in both stages. As expected the yeast attained a steady state in stage one and E. coli in stage two, but the yeast in the second stage had the same steady state population as the yeast in stage one. This means that the yeast did not grow at all in the second stage even though there was ample food supply for abundant growth of E. coli. This result was experienced over a range of dilution rates. Figure 36 illustrates a typical start-up of this system. The population in Stage I increased until a steady state was reached and the E. coli attained a steady state in Stage II. The yeast in Stage II initially grew beyond the level in the first stage, but after 6 hours yeast growth evidently stops and the population dilutes down to the same population as Stage I. At the time that the yeast in Stage II stopped growing the coliform was still growing quite readily. This means that there is absolutely no growth of yeast in the second stage.

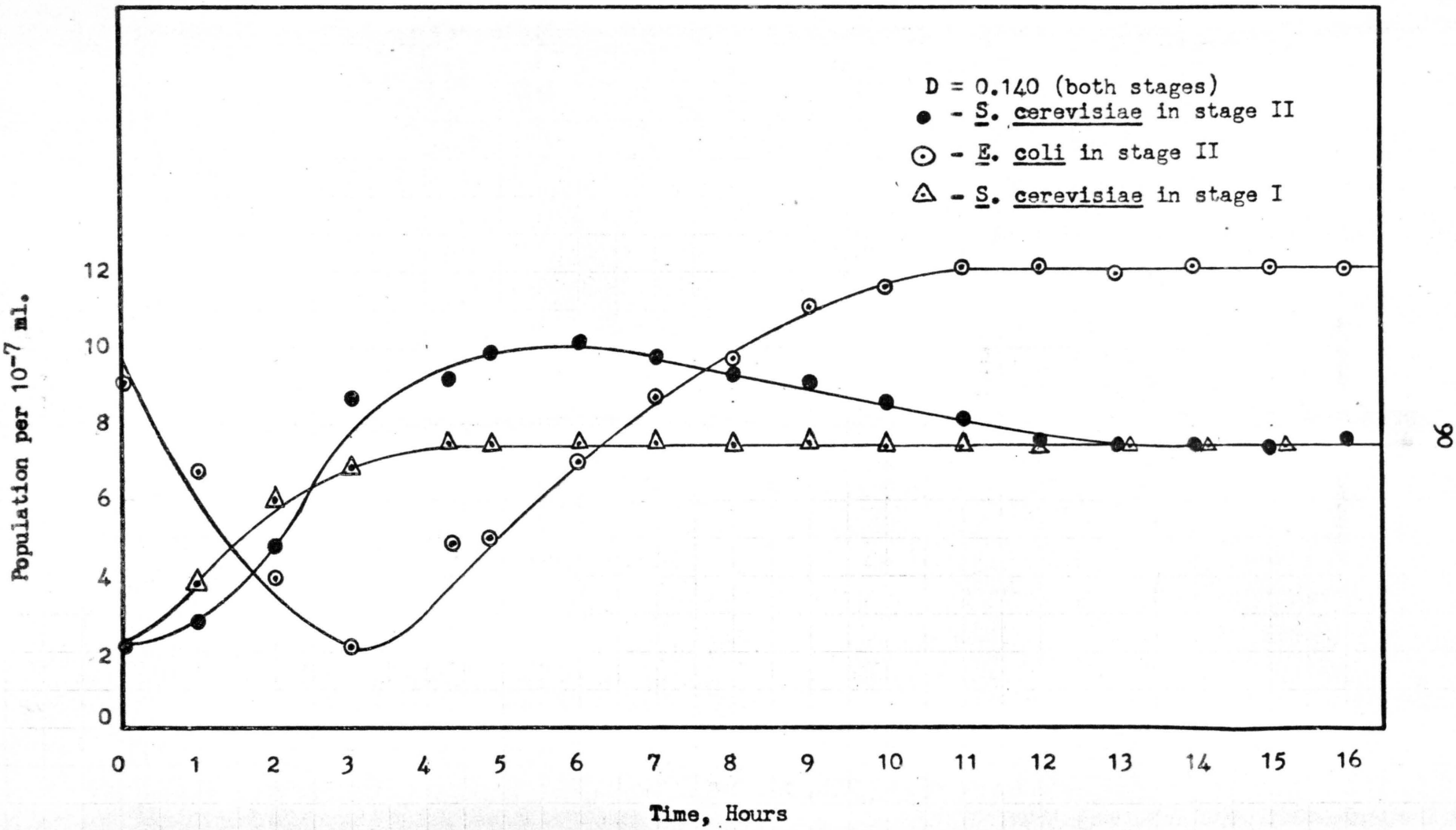


Fig. 36 Contamination of S. cerevisiae with E. coli in a two stage continuous culture system.

DISCUSSION

The continuous culture of microorganisms is an increasingly important technique to sanitary engineering. Most biological waste treatments operate as a continuous or semi-continuous culture of microorganisms, and to understand natural environments it is necessary to investigate the complexities of microbial interaction. Generally, the continuous flow technique will allow control over growth deqendent factors such as ph, concentration of nutrients, metabolic products, and dissolved oxygen which invariably change during the growth cycle in batch culture. These factors can usually be maintained constant and may be independently controlled in continuous culture. There have been cases where steady-states did not exist in continuous culture. The most widely known is the observation by Finn and Wilson (1954) of oscillation in a continuous culture, where the pH and population oscillated continuously. Also, Yeoh and Bungay (1965) have found that the mutualism interaction between P. vulgaris and B. polymixa never attained steady-states. Cassell, et al (1964) found that an activated sludge unit can exhibit wide fluctuations in various microbial species with time even though the feed rate and concentration are held constant.

However, in reviewing Shindala's data it was evident that for proper interpretation of mixed culture data the dynamics of pure cultures would have to be investigated. Also, a close review of Shindala's data revealed the possibility that the yeast S. cerevisiae does not follow the classic continuous culture theory.

The original paper which gave a theoretical basis for predicting steady-states was formulated by Herbert, et al (1956) based partially on the work of Monod (1942). In 1964, Schulze developed an extended theory based on experimental data which was similiar to Herbert's theory but accounted for the fact that the yield constant was different for different dilution rates. However, Herbert (1958) later cited several possible exceptions to the general theory and data presented in this paper is another exception to the general theory. In reviewing the literature this author has concluded that there are numerous exceptions and that a particular organism is more likely to be an exception to the rule than not. What I am primarily interested in is cell mass and different investigators have chosen to measure different variables such as dry weight, total nitrogen, or turbidity. I chose to measure cell numbers since rapid andprecise counts could be made electronically.

The population declined almost linearly with dilution for all concentrations of nitrogen limited medium. However, the glucose limited medium produced a dilution rate versus steady-state curve that consisted of a constant portion up to $D = 0.210$ hr.$^{-1}$ and then became linear until "washout" occured. The question raised here is why should the yeast have different growth dividing characteristics in different media. The investigation of cell dry weight and Kjeldahl nitrogen using the nitrogen limited culture resulted in very unusual relationships. The dry weight decreased linearly, but not at as great a rate as the population. This indicated that the average dry weight per cell was increasing with dilution rate. Also, the Kjeldahl nitrogen per

cell increased with dilution rate but at a greater rate than dry weight per cell. This suggests the possibility that the relative rates of protein and cell wall synthesis are different. In fact, the demonstrated increase in cell volume reinforces this possibility.

As shown in Figure 10 the electronic size distributions of the yeast not only indicated increasing size with dilution rate, but the distributions are very different. The cells with younger average age were larger and more uniform in size as indicated by the more peaked curve. The investigation of average cell size over a range of dilution rates indicates that the average cell size increases with dilution rate. Also, the average cell size increases with increased medium concentration as was illustrated in Figure 15. The curve showing average cell size versus dilution rate for a 0.40 concentration of medium one was very similiar to the one for 0.20 concentration except that at the higher concentration the curve is flatter. Experiments have shown that this type curve will become flatter at higher medium concentrations until it is virtually constant at very high concentrations.

Since the curves for dry weight per cell, Kjeldahl nitrogen per cell, and average cell size (equivalent diameter) are very similiar in shape there can be little doubt that the cell size increases dramatically with dilution rate in dilute media. Cell wall material makes up a high percentage of the dry weight of cells, and this gives rise to speculation that proteins are being synthesized at a much faster rate than cell wall material, as the dry weight per cell does not increase as does the protein nitrogen per cell. Also, the cell size

curves reflect volume more than diameter since the Coulter Counter actually measures volume. This indicates that intracellural materials (proteins) are being synthesized much faster than cell wall material and therefore the cell increases in size until enough material is produced for division. Actually, some cells were observed that were much larger than the average equivalent cell diameter at a particular flow rate. Sometimes a few cells were observed to be as much as 70 to 80 percent larger than the average cell, and several times cells in the seven to eight micron class were observed.

Response of microbial systems to abrupt changes in environmental factors have recently been of considerable interest. Yasuda and Mateles (1964) found that E. coli experiences a sudden increase in growth rate in response to a sudden change in dilution rate. Also, Herbert (1962) reported that microorganisms instantaneously adjusted their growth rate when abrupt changes are made in either the dilution rate or medium concentration.

The results of this research tend to agree with Herbert with regard to step changes in medium concentration or limiting nutrient. This is an instantaneous change in growth rate was experienced in response to a step change in ammonium sulfate. However, the response of S. cerevisiae to a step change in nongrowth limiting substrate, glucose, was found to be very interesting. The step change in glucose produced decaying oscillations with the initial and finalpopulations being identical. These oscillations are very interesting particularly since little time elapses before the response begins. Thus, glucose must

affect the control mechanisms for growth, but only in a transient manner. Furthermore, these mechanisms must operate rapidly because there was no apparent lag between the time the glucose was changed in the incoming medium and a growth response was noted. The control mechanisms must be very sensitive because glucose concentration in the flask could change but very slightly so soon after the change in feed. An interesting observation here is that it has been shown that glucose can trigger oscillations in yeast pyridine nucleotide (Chance, et al, 1964).

Oscillations initiated by dilution rate changes indicate the sensitivity of growth rate control mechanisms, since there was little time lag before the response. That is, little medium flowed at the new rate, and the yeast cells must have sensed small changes in their environment.

Preliminary experiments have shown that step changes in temperature and pH tend to cause oscillations in the yeast culture. These oscillations are not as interesting as the others because the step changes are more drastic. This means that the cells are sensing immediate changes and not small changes as in the step change in dilution rate.

Recent studies on ribosomes indicate that the cells in a steady state adjust the number of ribosomes and keep the number necessary which are believed to be working at full capacity. This is physiologically efficient and advantageous. When cells are transferred

from a poor medium permitting slow growth to a rich medium permitting fast growth, it was found that the increase of the rate of protein and DNA synthesis occurred only after the amount of RNA reached a level corresponding to the new faster growth rate (Neidhardt, 1961, Kennell and Magasanki, 1962). This means that the amount of ribosomal RNA in steady-state growth is a function of the growth rate. Thus when a continuous culture steady state is subjected to a disturbance, the adjustment of the number of the ribosomes occur first while the limiting nutrient accumulates. Therefore, there should be a lag in the growth rate. However, the experiments with step response indicated a very rapid increase in growth rate and then oscillated until a new steady state was established.

To insure that the oscillations did not reflect in cell dividing, or cell size, spot checks were made of electronic counts, chamber counts, dry weight and kjeldahl nitrogen near the peak of several oscillations. The results indicated that the oscillations are reflected in all of the variables measured. Also, the experiments indicated that steady-state populations were very stable and thus the oscillations in response to step changes are significant.

These complex responses to forcing inputs are not readily amenable to system analysis by conventional means. Frequency response analysis is superior to transient response analysis in that nonlinearities in the system are revealed as distortions of the output sine wave, and often acceptable analysis can be made despite nonlinearities in the input or output curve. Also, frequency response

analysis avoids the "Nyquist Folding" (analysis does not yield a unique solution) encountered in Fourier treatment of transient response data.

The dilution rate generator designed and built by Sutherland (1965) produced very good sine waves, and the output curves were of sufficient quality for this research. Also, the dilution rate generator was successfully arranged with two switches on the pivoting arm with one switch off when the other was on. This allowed the satisfactory production of substrate sine waves at a constant dilution rate.

The Bode plots for dilution rate indicated a third-order system with a quadratic second-order system added to a first-order system. The time constants obtained seem to be reasonable considering equipment and sampling techniques used. The Bode plot for response to medium concentration was also acceptable with two time constants being obtained. The largest of which represented the hydraulic constant of the culture vessel. The concentration of medium in the vessel was changing as a sine wave but 90° out of phase with the input sine wave. The cells respond to the concentration in the culture vessel and the output curve reflects this. However, this relationship is reflected in the Bode plot as another first-order system.

The attempts to sine force glucose and nitrogen concentration were met with little success. The failure of yeast to react to forcing of glucose can possibly be explained by the limits of the system utilized. That is, it is possible that the frequency range

of the generator is too low to investigate the probable frequencies required for the solution of this problem. The data obtained by forcing the ammonium concentration has not been explained at this time.

One of the most serious problems encountered with this work was the difficulty in fitting a sine wave to the outflow data. The Bode plots previously presented were obtained by hand-fitting sine waves to the outflow curves and estimating the amplitudes and phase lag. However, all future data will be fitted to a sine wave by use of a least squares fit computer program (Thompson, unpublished).

The mixed culture data indicated that there exists a critical concentration of E. coli which completely shuts off growth of S. cerevisiae. This critical population is independent of medium concentration. This suggests that E. coli produces some inhibatory factor that completely stops the growth of S. cerevisiae. This factor has not been isolated at this time, but several possible items have been eliminated. One of the first factors investigated was pH, as E. coli produces a number of acids which could possibly lower the pH to a level that would inhibit the growth of the yeast. However, the pH never dropped below 6.9 in any of these experiments, and was omitted as a possible answer.

The results obtained in this research indicate that the Coulter Counter is an excellent instrument for the rapid enumeration of microorganisms. It is possible to make measurements as often as one per minute. This speed proved to be an extremely valuable aid

in obtaining sine waves. Furthermore, size distribution curves for different growth conditions would have required highly tedious measurements by the other available methods. Actually, it would have been almost impossible to attempt this investigation without the use of the Coulter Counter.

The time constants are 8 and 40 minutes for dilution rate and 10 minutes for medium concentration. The larger time constant for response to medium concentration is simply the hydraulic time constant for the system. However, the smaller constant for medium concentration and the constants for dilution rate are constants for growth response for the yeast. The magnitude of these constants lead to speculation about the mechanisms for growth rate control. Enzymatic reactions and diffusion through cell wall membranes would be expected to take place in seconds or fractions of seconds. The processes which might take place in minutes would be those of protein accumulation, cell wall synthesis, and accumulation of genetic materials. Thus, it would seem that the control of cell growth rate would depend upon accumulation mechanisms. This is apparently in disagreement with other work (11, 25, 26) reporting an almost instantaneous response of growth rate to a change in dilution rate. However, the responses were highly complex and the initial step may have been activation of some very rapid control step followed by a depletion of reserves already accumulated. This would represent a high frequency outside the range of the frequency range of the present investigation.

CONCLUSIONS

1. The growth of S. cerevisiae in continuous culture does not follow classic continuous culture theory.
2. Average equivalent cell diameter, dry weight per cell, and Kjeldahl nitrogen per cell increased with dilution rate.
3. The ratio of rate of synthesis of cell wall material to rate of synthesis of proteins appears to decrease with increasing dilution rate.
4. The critical dilution rate, D_c, at which the yeast washed out decreased as the substrate concentration decreased.
5. Continuous cultures of S. cerevisiae are sensitive to abrupt changes in environmental factors such as glucose concentration, dilution rate, and temperature. Instantaneous abrupt changes (step changes) in dilution rate and glucose resulted in oscillations of yeast growth rate in a nitrogen limited medium. Oscillations were not observed in a glucose limited culture of S. cerevisiae.
6. Sinusoidal forcing of S. cerevisiae met with reasonable success and the equipment and techniques utilized in this research appears to be suitable for analysis of microbial systems.
7. Time constants for microbial systems may be obtained by use of the Bode Diagram with a minimum of mathematics. The time constants found for S. cerevisiae are:
    a. For response to dilution rate; 40 and 8 minutes.
    b. For response to medium concentration, 10 minutes.

8. The time constants from Bode plots seem to relate to mechanisms for the accumulation of cell constituents needed before cells can divide.

9. *E. coli* excretes an unknown inhibitory factor which can completely shut off growth of *S. cerevisiae*.

ACKNOWLEDGEMENT

The author would like to take this opportunity to express his appreciation to his thesis advisor, Dr. Henry R. Bungay III, for his encouragement, guidance, and constructive criticism; to Dr. William A. Parsons, for his advise and encouragement; to Dr. Noel R. Krieg, for his valuable suggestions; to Mr. Glenn Willard, for his invaluable aid during this research; and to my wife Nanna for her wonderful support during the entire course of this study and for typing this thesis.

Also, the author would like to take this opportunity to express his thanks to Dr. S. B. Row for allowing him time to pursue graduate work while employed by him; to the National Institute of Health, and the Ford Foundation for financial aid during this research; and to Mr. S. T. Moseley for allowing the author a teaching schedule that made part-time study feasible.

The vita has been removed from the scanned document

BIBLIOGRAPHY

1. Bartlett, M. C., "Continuous Antibiotics Fermentation," Ph. D. Dissertation, University of Michigan, 1958

2. Cassell, E. A., Sulzer, R. T., and Lamb, J. C. 3rd., Publication 94, School of Public Health, University of North Carolina, 1964

3. Chance, D., (Chairman) Symposium on Oscillatory Biochemical Responses, 150th Meeting, American Chemical Society, 1965

4. Chance, B., and Schoener, B., "Control of Oscillations of The DPNH Level in a Cell-Free Extract of Saccharomyces Carlsbergensis by 3' 5' Cyclic AMP," Biochem. Biophys. Res. Com., Vol. 17, No. 4, 1964

5. Curby, W., Swanton, D., Wallace, J., and Lind, H. E., "Determination of Minimal Sampling Interval for Electronic Counting of Microorganisms," Bacteriology Proceedures, G 64, 1964

6. Deysson, G., and Lau, N. T., "Utilization d'un Compteur Electronique de Particules pour L'Etude de la Croissance de Microorganisms," Annales Pharmaceutiques Francaises, Vol. 21, No. 4 pp. 275-285, 1963

7. Fiechter, A., and Ettlinger, A., Continuous Cultivation of Microorganisms, p. 245, New York, Academic Press, 1962

8. Finn, R. K., and Wilson, R. E., "Population Dynamics of A Continuous Propagator for Microorganisms," Vol. 2, No. 4, pp. 66-69, J. Agr. and Food Chem., 1954

9. Fuld, G. J., Mateles, R. I., and Kusmierek, B. W., Continuous Culture of Microorganisms, p. 54, New York, The MacMillan Co., 1961

10. Gerhardt, P., and Bartlett, M. C., "Continuous Industrial Fermentations," Advances in Applied Microbiology, Vol. I, pp. 215-260, New York, Academic Press, 1959

11. Gilley, J. W., and Bungay, H. R. 3rd., "Oscillatory Growth Rate Response of S. cerevisiae in Continuous Culture," Bio-technology and Bio-engineering, (Journal) In press

12. Gilley, J. W., and Bungay, H. R. 3rd., "Frequency Response Analysis of Yeast Growth Rate," Bio-technology and Bio-engineering, (Journal) In press

13. Golle, H. A., "Theoretical Considerations of a Continuous Culture System," Jour. Agr. Food Chem., Vol. 1, pp. 789-793, 1953

14. Herbert, D., Elsworth, R., and Telling, R. C., "The Continuous Culture of Bacteria, A Theoretical and Experimental Study," Jour. Gen. Microbiol., Vol. 14, pp. 601-622, 1956

15. Herbert D., "Continuous Culture of Microorganisms, Some Theoretical Aspects," Continuous Cultivation of Microorganisms, A Symposium, pp. 45-52, Prague, Czechoslovak Academy of Sciences, 1958

16. Herbert, D., Continuous Cultivation of Microorganisms, p. 43, Prague, Czechoslovak Academy of Sciences, 1962

17. Jacobson, R. L., "Determination of the Resolving Power of the Coulter Counter," Master Thesis, Virginia Polytechnic Institute, 1964

18. James, T. W., and Anderson, N. G., "Continuous Recording of Cell Number in Logarithmic and Synchronized Cultures," Science Vol. 142, pp. 1183-1184, November, 1963

19. Kennel, D., and Magasanik, B., "The Relation of Ribosome Content to the Rate of Enzyme Synthesis in Aerobacter Aerogenes," Biochem. Biophys. Acta, Vol. 55, pp. 139-151, 1961

20. Kubitschek, H. E., "Electronic Counting and Sizing of Bacteria," Nature, Vol. 182, pp.234-235, July, 1958

21. Lark, K. G., and Lark, C., "Changes During the Division Cycle in Bacterial Cell Wall Synthesis, Volume, and Ability to Concentrate Free Amino Acids," Biochem. Biophys. Acta, Vol. 43, pp. 520-530, September, 1960

22. Malek, I., "Development and Further Perspective of the Continuous-Flow Method of Cultivation of Microorganisms in Continuous Culture of Microorganisms," A Symp. Soc. Chem Ind. London, pp. 3-20, New York, The MacMillan Co., 1961

23. Mandelstam, J., "The Free Amino Acids in Growing and Non-Growing Populations of E. coli," Biochem. J., Vol. 69, p. 103, 1958

24. Manual of Microbiological Methods, p. 172, The Society of American Bacteriologists, New York, McGraw-Hill, 1957

25. Mateles, R. I., and Yasuda, T., "Response of Continuous Cultures of Escherichia coli to Disturbances," Talk given at 148th. meeting of the American Chemical Society, Chicago, Illinois, Sept., 1964

26. Mateles, R. I., Ryu, D. Y., and Yasuda, T., "Measurement of Unsteady State Growth Rates of Microorganisms," Nature, Vol. 208, No. 5001, p. 263, October, 1965

27. Mateles, R. I., Yasuda, T., and Goldthwaite, R. W., "Transients in Continuous Cultures," Abstracts of 145th. Meeting of the Am. Chem. Soc., New York, September, 1963

28. Monod, J., "Latechnique de culture continue theorie et applications," Ann. Inst. Pastuer, Vol. 79, pp. 390-410, 1956

29. Neidhardt, F. C., and Frankel, D. G., "Metabolic Regulation of RNA Synthesis in Bacteria," Cold Spring Harbor Symposia on Quantitative Biology, Vol. 26, pp 63-74, 1961

30. Novick, A., "Growth of Bacteria," Ann. Rev. Microbiol., Vol. 9, pp. 97-109, 1955

31. Novick, A., and Szilard, L., "Description of the Chemostat," Science, Vol. 112, pp. 715-716, 1950

32. Oldenburger, R., Frequency Response, New York, The MacMillan Co., 1956

33. Parker, R. B., and Snyder, M. N., "Interaction of the Oral Microbiota, A System for the Defined Study of Mixed Cultures," Proc. Soc. Exp. Biol., Vol. 108,p749, 1961

34. Schilling, G. D., Process Control and Dynamics, New York, Holt, Rinehart, and Winston, 1963

35. Schulze, K. L., "The Activated Sludge Process as a Continuous Flow Culture," Water and Sewage Works, pp. 526-538, December, 1964

36. Shindala, A., Bungay, H. R. 3rd., Krieg, N. R., and Culbert, K., "Mixed Culture Interactions, I. Commensalism of Proteus vulgaris with Saccharomyces cerevisiae in Continuous Culture," Journal of Bacteriology, Vol. 89:3, pp. 693-696, 1965

37. Shindala, A., "Growth in Mixed Cultures of Microorganisms," Ph. D. Thesis, Virginia Polytechnic Institute, 1964

38. Standard Methods for the Examination of Water and Waste Water, Eleventh Edition, p. 298, New York, American Public Health Assoc., 1960

39. Sutherland, E. R., and Bungay, H. R. 3rd., "Simulation of Sine Pumping With Pulse Pumping," American Institute of Chemical Engineers Journal, In Press

40. Swanton, E. M., Curby, W. A., and Lind, H. E., "Experiences With the Coulter Counter in Bacteriology," Appl. Microbiol., Vol. 10(5), pp. 480-485, 1962

41. Thompson, W. O., Unpublished (Available upon request from the Civil Engineering Dept., Virginia Polytechnic Institute, Blacksburg, Virginia

42. Williams, T. J., Systems Engineering for the Process Industries, New York, McGraw-Hill, 1961

43. Yeoh, H. T., and Bungay, H. R. 3rd.,(Personal communication)

APPENDIX

THEORY OF THE COULTER COUNTER

The Coulter Counter determines the number and size of particles suspended in an electrically conductive liquid. The suspension is made to flow through a small aperture in a glass wall that separates the suspension into two electrically isolated portions. Electrodes are placed in the suspension on either side of the wall and connected to a direct current supply. Resistance to current passing through the suspension between the electrodes will depend almost entirely on the resistance in the small aperture. In the absence of particles the resistance will remain constant. However, when a particle passes through the aperture the resistance rises because of the reduction of the volume of electrolyte. The particle concentration is diluted to a level such that usually only one particle passed through the aperture at a time. Each particle passage displaces electrolyte within the aperture, momentarily changing the resistance between the electrodes and producing a current pulse of magnitude proportional to particle volume. The voltage pulses are amplified and sent through a gating circuit having an adjustable threshold level. All voltage pulses equal to or greater than the threshold level activates the counter driver, and pulse is counted. The circuit is also linked into an oscilliscope screen, and a brightening of all pulse segments above the threshold setting aids the operator in adjusting the instrument.

The pulse height and instrument response are essentially proportional to particle volume and to fluid resistivity for particles up to 30 or 40 percent of aperture diameter. By use of a suitable conversion factor the particle volume can be related to the diameter of a sphere of the same volume. Adjustment of the threshold level above which pulses are counted enables the determination of particle size distribution. The Model A Counter used here is a seven-decade digital instrument capable of counting pulses at a rate of 5,000 per second. Sample dilution was selected to provide the optimum rate of flow of particles through the aperture without exceeding the safe coincidence correction values. The conductivity of the diluent can be adjusted to provide to provide the optimum electric current flow through the aperture, but membrane-filtered 0.9 percent saline was used during this research. The variables to be considered in particle size analysis are: particle concentration, the conductivity of the electrolyte, counting time, and the aperture diameter. Particle diameters should be between 1½ percent and 30 percent of the aperture diameter, with 1 percent to 40 percent being extremes(20:1 to 40:1 on diametric basis, or 8,000:1 to 64,000:1 on volumetric basis). This research employed both commercial orifices and orifices made in our laboratory (see Shindala, 1964, p. 98).

Calibration of the equipment is carried out by means of a suspension of particles of known diameters. There are two controls used in this calibration; the dial adjusting the aperture current and the dial adjusting the threshold voltage for pulse counting.

The basic construction of the Coulter Counter is shown in Fig. 37. One electrode is placed in the beaker containing the sample. Into the same beaker is placed a glass tube, sealed from the contents of the beaker except at the point where the aperture is located. The second electrode is contained in this tube, immersed in the suspension. To the top of the tube is connected a mercury manometer and, through a stop cock, to a vacuum pump. The vacuum pump runs continuously and provides a negative pressure of about 11 lb./sq. inch. When the stop cock is opened, mercury is drawn from the manometer towards the tube containing the orifice. When the stop cock is closed, the mercury flows back to its original balanced position, at the same time drawing samples from the beaker into the tube by way of the aperture. Two contacts inserted in the wall of the manometer provide a means of insuring that the number of particles in an exact volume (0.05 cubic centimeters) of the sample is counted as it passes through the aperture, counting being started when the mercury contacts the first electrode and stopped when it contacts the second electrode. Thus, the number of particles is always counted in equal volumes of fluids, and this volume is smoothly flowing both at the start and at the finish of the count. A home made timer, the same as reported by Shindala (1964) p. 94 calibrated to give a selected time period of about 10 seconds was utilized in a portion of this research along with the manometer method. The variations between counts when the timer was used compared with that obtained with the 50-micron orifice using the manometer proved that the timer was completely satisfactory.

As well as being counted electronically, the voltage pulses are displayed on a cathode ray tube. The pattern of pulses shown serves as a guide for sample dilution and for dial settings and also acts as a monitor of instrument performance. The threshold level above which pulses are recorded for counting is displayed on the screen as a brightness modulation. Pulses above the threshold level are brighter than those below it.

During the course of the analysis it is sometimes possible for an extra large particle to lodge in the orifice, thus giving an erroneous count. A microscope provided for orifice examination was useless because of the tiny orifice sizes. Actually, plugging did not provide any serious problems during the entire course of this investigation.

The high number of particles counted provides high statistical accuracy, but a correction for particle coincidence in the orifice was not needed at the dilutions employed. Also, the fact that the diameter of a particle is proportional to the cube root of its volume means that errors in volume measurement are reduced to their cube root when the measurement is expressed as an equivalent diameter. Consequently, the makers of the instrument claim that diameter increments of 1 percent can be differentiated.

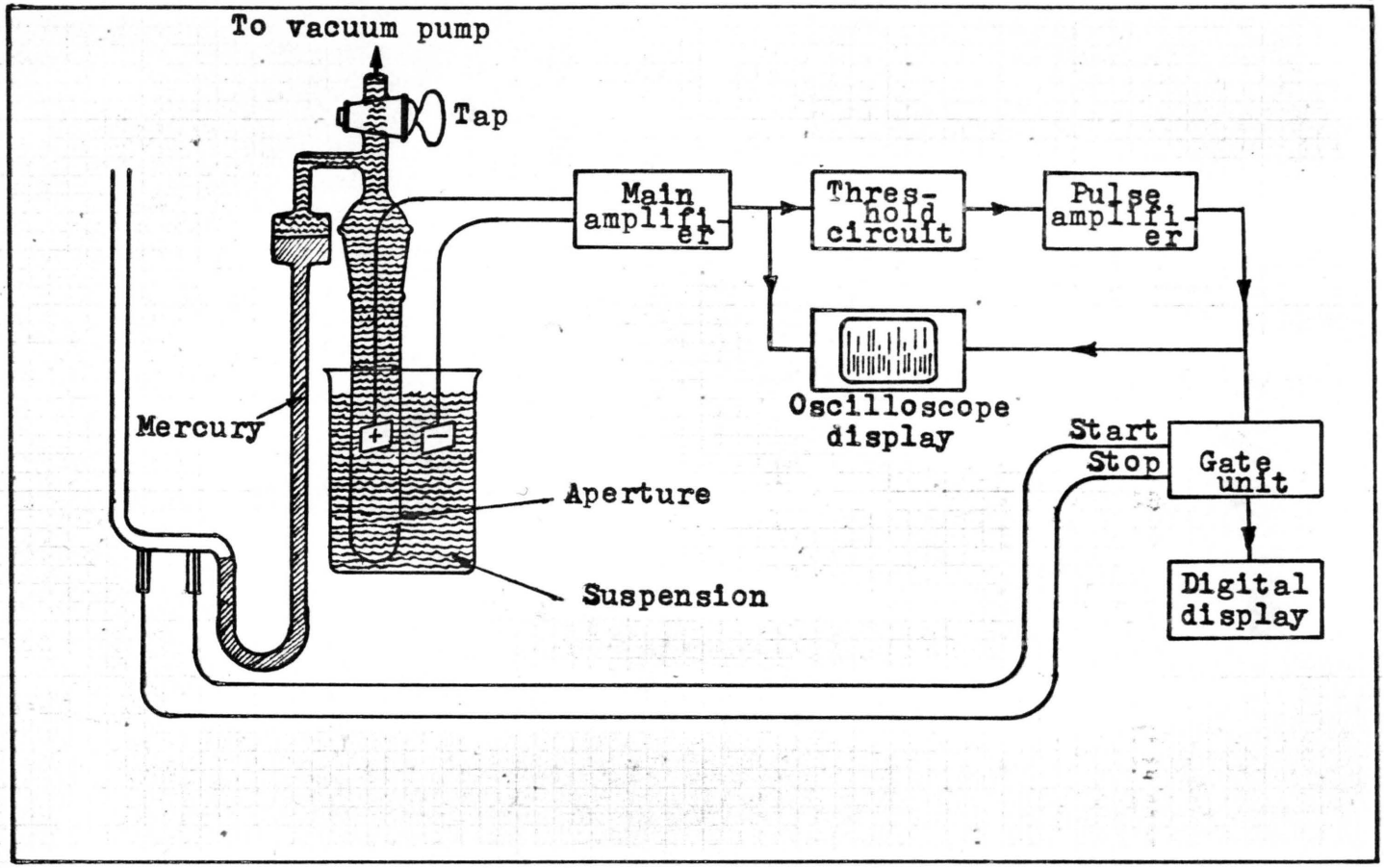


FIG. 37 BASIC CONSTRUCTION OF THE COULTER COUNTER

ABSTRACT

Pure cultures of the yeast S. cerevisiae do not follow the classical continuous culture theory when tested in a variety of chemostats. Using a Coulter Counter, yeast populations were sized and enumerated electronically over a range of dilution rates. Total dry weight and Kjeldahl nitrogen per cell were determined for a variety of dilution rates. From these data it was shown that the yeast definitely increased in size as the dilution rate increased. Also, it appeared that the ratio of rate of synthesis of cell wall material to the synthesis of proteins decreased as the dilution rate increased.

Step changes in various environmental factors (dilution rate, medium concentration, glucose concentration, ammonium sulfate, and temperature) indicated that yeast populations may not show a smooth transition from the initial to the final steady states. Decaying oscillations of the yeast population were observed in response to step changes in dilution rate and glucose concentrations. Step changes in ammonium sulfate or in total medium concentrations resulted in smooth responses to a new population level.

To investigate the complex responses to dilution rate and glucose a frequency response analysis was employed. The growth of S. cerevisiae varied in a sinusoidal fashion in response to sinusoidal forcing of dilution rate and medium concentration.

Bode diagrams were prepared for yeast response to dilution rate and medium concentration. Time constants were determined from the Bode diagrams. The time constants were in the order of several minutes and possible relations to growth control mechanisms can be postulated. The time constants probably pertain to accumulation of intracellular materials.

A two-stage continuous culture system was built to investigate the interaction of E. coli and S. cerevisiae. By feeding a continuous pure yeast culture from the first stage into the mixed culture second stage it was found that the E. coli apparently produces a substance that inhibited the growth of yeast.