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Kinetics of Growth and Substrate Uptake in a Biological Film System

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The rates of growth and substrate uptake in a biological film continuous-flow reactor were studied. The experiments were performed with high fluid velocities to bring the reactor operation to the reaction-controlled regime, thus avoiding external diffusional resistances. The glucose uptake experiments were performed with small film thicknesses so that full substrate penetration within the entire film thickness could be obtained. In this way, the catalyst effectiveness factor was 1.0 and the observed rate was the true, or intrinsic, rate. The results of the experiments indicate that both the intrinsic rate of substrate uptake and the rate of film growth are independent of the substrate concentration remaining in the reactor (zero-order reactions). However, the value of the initial substrate concentration when the film is in the early stages of growth defines the magnitude of both the rate of uptake and growth. This effect of the initial substrate concentration follows a saturation-function pattern.

Most of the research carried out in the fields of applied biology and environmental engineering concerning the kinetics of substrate removal by microorganisms has been directed to the case of suspensions of organisms in batch and continuous cultures. The bulk of the existing literature assumes that the microbial culture is a homogeneous suspension, i.e., that the substrate consumption process is a homogeneous reaction. However, in recent years several investigators (8; C. R. Baillod, Ph.D. thesis, Univ. of Wisconsin, Madison, 1968; W. Gulevich, Ph.D. thesis, Johns Hopkins Univ., Baltimore, 1967; J. A. Mueller, Ph.D. thesis, Univ. of Wisconsin, Madison, 1966) have demonstrated that mass transfer resistances play an important role in the kinetics of biological oxidation of organic matter.

In the case of biological films, it has been shown that the substrate consumption reaction is affected by both external and internal diffusion. Several investigators (6; 9; Gulevich, thesis, 1967; E. J. LaMotta, submitted for publication, 1975) have demonstrated that fluid velocity has a positive effect on the rate of substrate uptake. As presented elsewhere (LaMotta, submitted for publication), the uptake rate can be increased by increasing the fluid velocity, until the process becomes reaction-controlled; in this regime, the rate is no longer affected by the liquid velocity.

The effect of film thickness on the substrate uptake rate has also been analyzed in the literature (9; 14; R. C. Hoehn, Ph.D. thesis, Univ. of Missouri, Columbia, 1970; LaMotta, submitted for publication). LaMotta (submitted for publication) has shown that, unless there is complete substrate penetration within the biological film and external diffusional resistances have been eliminated, the true rate cannot be observed. These conditions can be satisfied by introducing a sufficiently high fluid velocity and by dealing with small film thickness.

With regard to the order of the substrate uptake reaction, there are several different opinions that can be found in the published literature. However, most of the rate expressions in current use are of the zero-order type or of the Monod type.

The evidence presented in the literature supports the hypothesis of zero-order uptake rates by microorganisms. The investigation described herein also demonstrates that zero-order kinetics adequately explains the substrate rate by biological films.

The classic studies of Monod (11) on the growth of pure batch cultures of *Bacillus coli* have been used extensively in the fields of applied biology, fermentation engineering, and environmental engineering. The resulting equation,

$$\mu = (\mu_{max})\frac{S}{K_s + S} \quad (1)$$

where μ is the specific growth rate, μ_{max} is the asymptote of the curve, K_s is the numerical constant, and S is the substrate concentration at time t in a batch reactor, relates the specific

growth rate and the concentration of substrate remaining in a batch system, when substrate S is the limiting nutrient.

Although Monod chose an equation of the form of equation 1 because it was "... both convenient and logical to adopt a hyperbolic equation... similar to an adsorption isotherm or to the Michaelis equation" (11), S. Ghosh (Ph.D. thesis, Georgia Institute of Technology, Atlanta, 1969) and F. E. Stratton (Ph.D. thesis, Stanford Univ., Stanford, Calif., 1966) have derived it from considerations of the Michaelis-Menten relationship for the rate of substrate-enzyme reaction.

A massive experimental effort has been undertaken by Gaudy and co-workers (2-5, 13, 14) to test the applicability of the equation of Monod to both batch and continuous cultures of microorganisms. Their results, obtained with both pure and heterogeneous batch cultures of microorganisms, indicate that μ is controlled in batch systems by the initial substrate concentration, S_0, and that the effect of S_0 on μ can be described by an expression of the form of equation 1 in which S (instantaneous) is replaced by S_0 (cf. reference 3), as indicated in equation 2,

$$\mu = \mu_{max} \frac{S_0}{K + S_0} \quad (2)$$

This behavior was confirmed in several tests with a wide variety of substrates (including sewage) and for both pure cultures and heterogeneous populations.

With regard to the effect of substrate concentration on the rates of film growth and substrate uptake in biological film systems, similar considerations to those discussed above can be made. Regardless of the type of reactor in which the film is growing, film development takes place under affixed conditions. This implies that if the reactor is a continuous-flow reactor and film growth is to start at time zero, the influent concentration of substrate will determine the number of permeation sites set by the cells to attain a balanced growth according to the amount of substrate externally available. This fixed number of active sites for uptake will define the magnitude of the rates of substrate uptake and film growth. Film accumulation will proceed at a constant rate determined by the magnitude of the concentration of substrate in the influent stream (a parameter that can be kept at a constant level), following a relationship similar to equation 2, i.e.,

$$\tilde{\mu} = \tilde{\mu}_{max} \frac{S_i}{K_s + S_i} \quad (3)$$

in which $\tilde{\mu}$ is the specific film growth rate (reciprocal seconds), $\tilde{\mu}_{max}$ is the maximum film growth rate, K_s is the numerical constant, and S_i is the influent substrate concentration.

At each level of influent substrate concentration, the number of film active sites will remain constant, yielding constant substrate uptake and film growth rates. This number of film active sites will not be affected by decreases in reactor substrate concentration that result from increasing biomass, until the concentration of nutrients becomes too low and a declining growth phase will take place.

With these concepts in mind, the objectives of this investigation were to (i) observe the intrinsic rate of the substrate consumption reaction by biological films, (ii) observe the kinetics of the film accumulation process, and (iii) observe the affect of the influent substrate concentration on the rates of substrate uptake and film growth.

MATERIALS AND METHODS

All experiments were performed under aerobic conditions in a continuous-flow laboratory reactor similar to that used by Kornegay and Andrews (9). It consisted of two concentric cylinders of different diameter, the inner one being stationary and the outer rotating about its axis. A glucose solution containing sufficient mineral nutrients flowed continuously through the annular space at a rate of 100 ml/min. The concentration of glucose in the influent stream was given several values (from 2 to 200 mg/liter) to test the effect of this parameter on the intrinsic uptake rate and on the rate of film accumulation. Glucose concentration was determined according to a modified Glucostat procedure, which is described in detail elsewhere (E. J. LaMotta, Ph.D. thesis, Univ. of North Carolina, Chapel Hill, 1974).

Film thickness was determined by means of two removable slides provided in the inner cylinder and four plugs located at different levels on the rotating cylinder wall. The thickness of the film adhering to the slide (or the plug) was measured by placing the sample on the stage of a microscope provided with a graduated micrometer. By focusing first at the top of the film, and then at the bottom of the film, the difference between the two readings gave the film thickness.

Before the series of experiments was started, the reactor contents were inoculated with film samples collected from one of the trickling filters of the Chapel Hill waste water treatment plant, and continuous operation was immediately started using a low flow rate (0.167 cm^3/s). The operation of the system was continued in this fashion for several days; later, gradual increases in the flow rate and decreases in the concentration of glucose were introduced until a flow of a 2.8×10^{-8} mol of glucose solution per cm^3 (5 mg/liter) at 1.667 cm^3/s (100 ml/min) was established.

The development of a stable film was a long process; the first films obtained did not adhere well to

the plastic walls and sloughed off as a whole. The acclimation of the microorganisms to the adverse hydraulic shearing conditions required approximately 1 month, after which period the film was extremely resistant to shear. Microscopy examination of the film under these conditions revealed the presence of free-swimming protozoa and a network of filamentous microorganisms, both characteristic of biological films.

Measurement of the specific film growth rate. The rate of film growth on the walls of the cylinders can be established by setting up a mass balance on the microorganisms present in the reactor. Using film volume as the parameter to measure film accumulation, it can be hypothesized that the rate of film growth is directly proportional to film volume, i.e.,

$$\bar{r}_g = \bar{\mu} \cdot V \tag{4}$$

where $\bar{r}_g$ is the rate of film accumulation (cubic centimeters of film per second), $\bar{\mu}$ is the specific film growth rate constant (reciprocal seconds), V is the film volume ($A \cdot \delta$, [cubic centimeters]), δ is the film thickness (centimeters), and A is the film area (square centimeters).

Equation 4 assumes that film density is constant, i.e., that film mass is proportional to film volume; this assumption is supported by the observations of Kornegay and Andrews (9) from their experiments with films using glucose as the only source of carbon. However, Hoehn and Ray (7) have recently published the results of their experiments with films grown in a medium whose constituents included (per liter) 700 mg of bacteriological nutrient broth, 150 mg of hydrated sodium acetate, and 150 mg of glucose, pointing out that film density (dry mass per unit volume) is not constant throughout the entire film thickness range. Whether this discrepancy is due to differences in the growth media used by these two investigators remains to be ascertained. As reported later, limited measurements of concentration of adenosine 5'-triphosphate (ATP) per unit of film area, performed by me, revealed a direct proportionality between film-active biomass and film volume. Hence the assumption on which equation 4 is based is reasonable.

The mass balance equation during the early stages of film growth, i.e., when suspended microorganisms cannot be detected in significant amounts, would be

$$\text{Accumulation} = \text{input} - \text{output} + \text{growth}$$

$$\frac{dV}{dt} = 0 - 0 + \bar{\mu}V \tag{5}$$

Integration of equation 5 yields

$$\int_{r_0}^{r} DV = \bar{\mu} \int_0^t dt$$

whose solution, expressed in terms of film thickness, is

$$\ln \frac{\delta}{\delta_0} = \bar{\mu}t \tag{6}$$

According to this equation, a plot of $\ln\delta$ versus t should yield a straight line with slope $\bar{\mu}$. This slope can be easily determined by graphically measuring the time required for the film to double its thickness, t_d, and using the following relationship derived from Equation 6:

$$\bar{\mu} = \frac{\ln 2}{t_d} = \frac{0.693}{t_d} \tag{7}$$

Once the degree of depletion of substrate and hydraulic shear impose sufficient stress on the film as to preclude indefinite exponential growth, the film accumulation curve is expected to level off following the characteristic growth pattern observed in batch cultures of suspended microorganisms.

Measurement of the specific substrate uptake rate. As mentioned before, in measuring the substrate uptake rate, care must be taken to eliminate the masking effects of diffusional resistances, both external and internal. To measure the true reaction order, the system must be operated in the kinetic regime and the film thickness must be maintained at levels that yield an effectiveness factor close to 1.0. High rotational speeds (greater than 150 rpm) and thin films that allowed complete substrate penetration throughout the entire film thickness resulted in the desired conditions.

In these circumstances, a mass balance on substrate is

$$V_L \frac{dS_b}{dt} = QS_i - QS_e - r_v \cdot A \cdot \delta \tag{8}$$

where V_L is the volume of liquid solution in the reactor (cubic centimeters), S_b is the substrate concentration in the bulk of the solution (moles/cubic centimeter of solution), S_i is the influent substrate concentration (moles/cubic centimeter of solution), S_e is the effluent substrate concentration (moles/cubic centimeter of solution), t is time (seconds), r_v is the true rate of substrate uptake per unit of film volume, (moles/second per cubic centimeter of film), A is the film surface area (square centimeters), δ is film thickness (centimeters), and Q is the volumetric flow rate of substrate solution (cubic centimeters/second).

Under steady-state conditions, and with complete mixing of the fluid (see LaMotta, thesis, 1974, for the demonstration of the validity of these assumptions), equation 8 yields

$$r_v = \frac{Q}{A}(S_i - S_e) \tag{9}$$

Equation 9 allows the calculation of the true substrate uptake per unit of film volume from a knowledge of measurable parameters.

The effect of influent substrate concentration on the rates of growth and substrate uptake was tested by using eight different values of S_i in correspondingly different runs.

The relationship between the rate of film growth and the substrate uptake rate can be expected to follow the simple relation

$$\frac{\bar{\mu}}{r_v} = Y \tag{10}$$

where Y is the yield coefficient (cubic centimeters of film per mole).

RESULTS

Film growth pattern. Typical film growth curves are shown in Fig. 1, demonstrating that film accumulation throughout time follows an exponential pattern such as that described by equation 6. It can be seen that the slope of the straight line portion of the curve, i.e., $\tilde{\mu}$, increases as influent substrate concentration increases. This point is illustrated in Fig. 2, in which all the values of $\tilde{\mu}$ are plotted as a function of S_i. It can be seen that the observations follow the pattern described by equation 3, thus supporting the assertion made earlier that the influent substrate concentration is the factor that determines the magnitude of the rate of film growth.

To find the numerical values of the parameters, $\tilde{\mu}_{max}$ and K_s, which would best fit equation 3 to the observed data, two different statistical procedures can be used. First, a linearization of the graph can be obtained by using the traditional Lineweaver-Burk plot (see Fig. 3), and a linear regression analysis would provide the values of the unknown parameters. One of the disadvantages of this method is that it is sensitive to small errors in the low-concentration range. Besides, the least-square regression line using these scales will not necessarily provide the best-fit curve in the plot $\tilde{\mu}$ versus S_i. This curve can be obtained using a second statistical procedure, namely, a nonlinear regres-

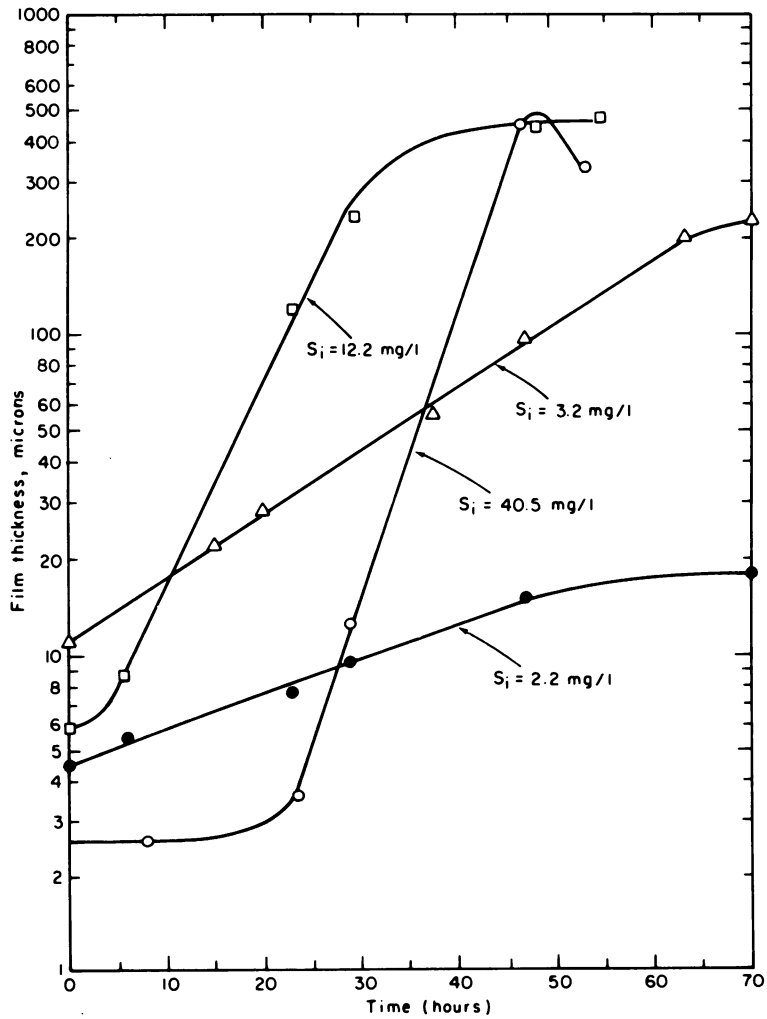

FIG. 1. *Typical film growth curves for several influent glucose concentration.*

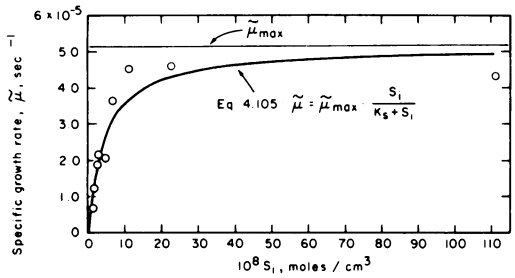

FIG. 2. *Effect of influent glucose concentration on the specific growth rate, $\tilde{\mu}$. The line of best fit was found by a nonlinear regression analysis yielding $\tilde{\mu}_{max} = 5.142 \times 10^{-5}$ per s and $K_s = 4.395 \times 10^{-8}$ mol/cm^3.*

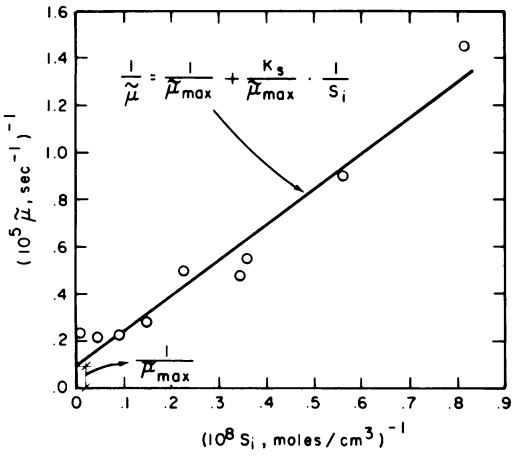

FIG. 3. *Lineweaver-Burk plot of the same data as Fig. 2. The linear regression analysis yields $\tilde{\mu}_{max} = 10.59 \times 10^{-5}$ per s and $K_s = 15.98 \times 10^{-8}$ mol/cm^3.*

sion analysis on the data. Both types of analysis were run by using computer programs prepared by the Department of Biostatistics of the University of North Carolina at Chapel Hill. Given the form of the function, with its parameters and variables, both regression analysis programs provide the numerical values of the parameters, such that the sum of the squares of the residuals is a minimum. The linear regression program is based on standard regression techniques, such as those described by Draper and Smith (1), whereas the nonlinear regression analysis uses an iteration procedure similar to that described by Nelder and Mead (12).

The numerical values of the parameters, as provided by this analysis, are the following: nonlinear regression, $\tilde{\mu}_{max} = 5.142 \times 10^{-5}$/s and $K_s = 4.395 \times 10^{-8}$ (mol/cm^3); linear regression (Lineweaver-Burk linearization), $\tilde{\mu}_{max} = 10.59 \times 10^{-5}$/s and $K_s = 15.98 \times 10^{-8}$ (mol/cm^3).

Although more experimental data are needed in the intermediate-concentration range, an analysis of Fig. 2 shows that a more realistic value of $\tilde{\mu}_{max}$ is that given by the nonlinear regression analysis. As stated before, the Lineweaver-Burk plot distorts the actual experimental observations, leading to erroneous estimates of the parameters. Therefore, the values provided by the nonlinear regression analysis will be adopted here.

Intrinsic glucose uptake rate. The values of the intrinsic glucose uptake rates obtained from eight different influent glucose concentrations are presented in Fig. 4a through c; it is seen that the intrinsic rate does not show any dependence on the bulk-liquid glucose concentration, thus supporting the hypothesized zero-order intrinsic kinetics ($r_r = k_r$, a constant).

If attention is paid to the magnitude of the intrinsic uptake rate, k_r, it will be seen that it increases as influent glucose concentration increases. This can be more clearly appreciated in Fig. 5, which is a plot of k_r versus S_i. Again, a saturation function, of the form of equation 11, i.e.,

$$k_r = k_{r_{max}} \frac{S_i}{K_s + S_i} \tag{11}$$

provides a satisfactory fit to the data. The values of $k_{r_{max}}$ and K_s were obtained by using the same statistical procedures described in the previous section, and they are: from nonlinear regression, $k_{r_{max}} = 8.03 \times 10^{-8}$ (mol/s per cm^3 of film) and $K_s = 1.11 \times 10^{-7}$ (mol/cm^3); from linear regression, $k_{r_{max}} = 6.21 \times 10^{-8}$ (mol/s per cm^3 of film) and $K_s = 6.8 \times 10^{-8}$ (mol/cm^3).

Using the same considerations presented in the case of equation 3, it may be concluded that the parameters estimated from the nonlinear regression analysis are more realistic than those estimated from linearization of the data (see Fig. 6). However, the numerical values of $k_{r_{max}}$ and K_s cannot be considered reliable since the analysis was performed on limited experimental information.

The validity of equation 10 is tested in Fig. 7, which is a plot of the values of k_r versus $\tilde{\mu}$. It can be seen that equation 10 provides a good fit through most of the points. The best-fit straight line forced through the origin of coordinates is $k_r = 0.952 \times 10^{-3}\ \tilde{\mu}$ (mol/s per cm^3 of film). The yield coefficient, as given from equation 10, would be $Y = (1.05 \times 10^3)$ (cm^3 of film)/(mol of glucose). This value predicts reasonably well the thickness of film observed in this investigation under each substrate concentration.

Film activity measurements. As pointed out previously, the theoretical zero-order model for

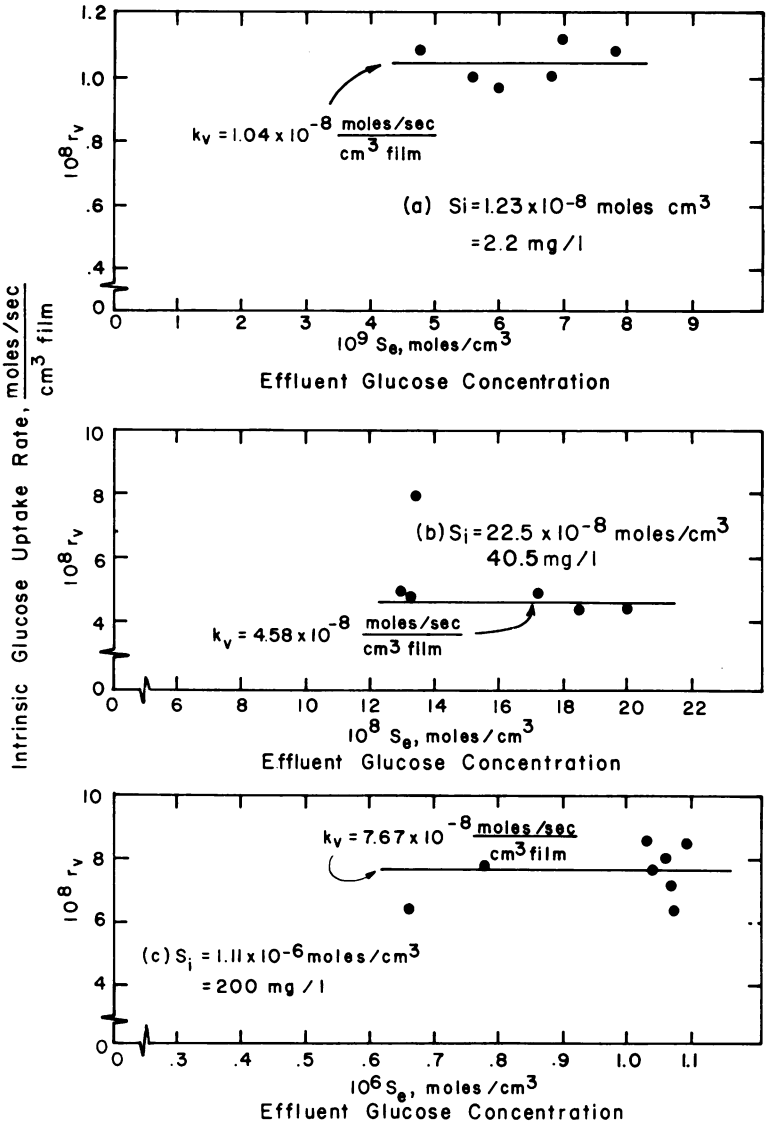

FIG. 4. *Zero-order relationship between intrinsic uptake and effluent glucose concentration.*

glucose removal in a biological film system implies that substrate is depleted in the superficial film layers; in this way, glucose should not be detected at depths greater than the depth of substrate penetration. However, it is possible that the substrate consumption reaction deviates from zero-order kinetics at the low-concentration range attained in the deeper layers of film; in this case the actual depth of penetration will be different from the theoretical zero-order critical depth, δ_c. Furthermore, by-products of the glucose metabolism carried out in the superficial layers can diffuse into the deeper film strata providing additional sources of food. Nevertheless, it is conceivable that the basal microorganisms will suffer from substrate starvation leading to the accumulation of dead organic matter in the deep layers of film.

In this situation, it is of interest to measure the viability of the film biomass to see if the assumption of proportionality between film volume and film active mass can be supported. The method for measuring film viability consisted of determining the concentration of ATP using the JRB-ATP photometer.

Fig. 8 is a plot of the values of ATP mass per unit of film area versus film thickness gathered from several runs under S_i = 200 mg/liter and

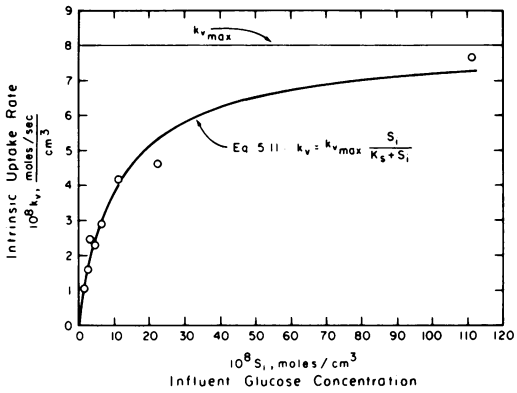

FIG. 5. *Effect of influent glucose concentration on the intrinsic uptake rate, k_v. The line of best fit was found by a nonlinear regression analysis yielding $k_{v\,max}$ = 8.03 × 10^{-8} mol/s per cm³ of film and K_s = 1.11 × 10^{-7} mol/cm³.*

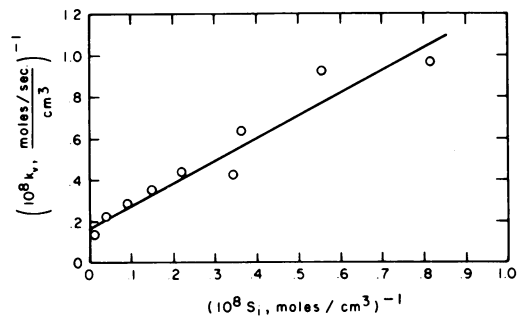

FIG. 6. *Lineweaver-Burk plot of the same data as Fig. 5. The linear regression analysis yields $k_{v\,max}$ = 6.21 × 10^{-8} mol/s per cm³ of film and K_s = 6.8 × 10^{-8} mol/cm³.*

a few points determined in preliminary tests when S_i = 5 mg/liter. It shows that the mass of ATP increased approximately proportionally to film thickness up to ca. 320 μm; for film thickness greater than 320 μm, the mass of ATP per unit area remained approximately constant. These two regions would seem to indicate that, once a certain film thickness has been exceeded, a portion of the film consists of nonliving organic matter.

The important point shown in Fig. 8 is that film activity remained approximately proportional to film thickness in a thickness range exceeding the theoretical zero-order depth of glucose penetration. This finding supports the assumption made earlier that film volume is proportional to active film mass; thus, expressing the rate of film growth and the rate of substrate consumption in terms of film volume rather than film mass is justified.

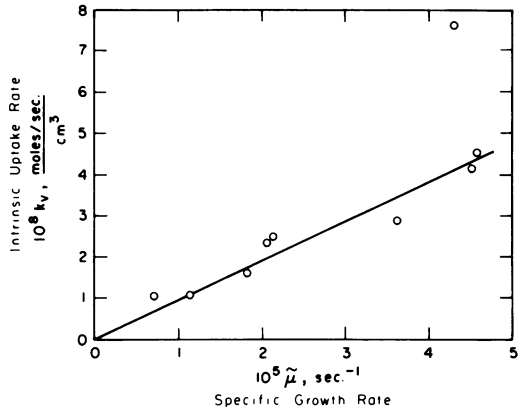

FIG. 7. *Relationship between intrinsic uptake rate and specific growth rate. The regression line was forced through the origin of coordinates, yielding k_v = 0.952 × 10^{-3} $\tilde{\mu}$ (equation 10).*

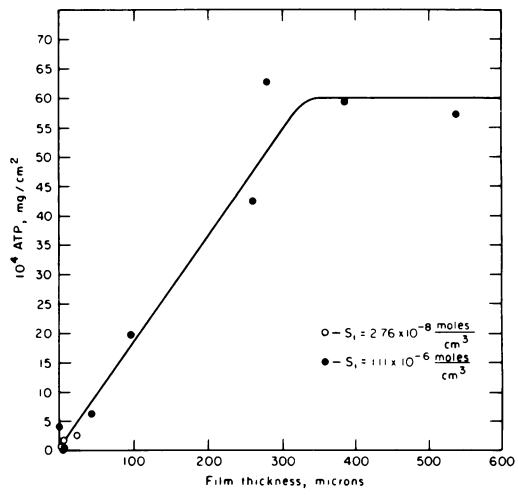

FIG. 8. *Relationship between mass of ATP per unit film area and film thickness.*

DISCUSSION

The results presented in the previous section demonstrate that the specific rate of film growth is independent of the concentration of substrate remaining in the reactor. Referring to Fig. 1, for S_i = 1.23 × 10^{-8} mol/cm³ (2.2 mg/liter), the effluent glucose concentration (which is equal to the concentration in the liquid bulk) after 29 h was measured to be S_e = 2.4 × 10^{-9} mol/cm³ (0.43 mg/liter), and exponential growth still was occurring. For S_i = 2.25 × 10^{-7} mol/cm³ (40.5 mg/liter), exponential growth ceased after 46.5 h, namely, when the effluent glucose concentration had decreased to 3.2 ×

10^{-8} mol/cm^3 (5.8 mg/liter); in other words, within a wide range of glucose concentration in the bulk liquid (from 40.5 to 5.8 mg/liter), exponential growth was not affected by glucose concentration. These observations support the hypothesis presented previously that the rate of film growth is not affected by decreases in reactor substrate concentration. Once the film microorganisms establish an internal steady state determined by the initial conditions of food availability, the rate of growth will not change despite significant reductions in the substrate concentration, until critical environmental conditions preclude further exponential film accumulation.

The effect of substrate concentration initially available on the magnitude of film growth rate is clearly seen in Fig. 2. Growth rate increases dramatically as initial substrate concentration is increased from low values up to ca. 10×10^{-8} mol/cm^3. Beyond this value, the rate of film growth becomes insensitive to changes in the influent glucose concentration. This phenomenon can be explained from the following considerations: first, a saturation of the cell active sites would produce a maximum substrate utilization that would be reflected in a maximum rate of film growth and, second, the high hydraulic shear conditions would additionally impose a limit on the growth pattern.

With regard to the specific substrate uptake rate, Fig. 4 clearly demonstrates that the rate follows a zero-order rate law. However, the concentration of substrate initially present when the film is in the early stages of development determines the magnitude of the rate of substrate uptake, and consequently the rate of film growth.

It is worthwhile emphasizing the fact that the measurements of the true (or intrinsic) rate of substrate uptake were performed under reaction-controlled conditions (obtained with high fluid velocities) and negligible internal-diffusion effects (obtained with small film thicknesses). Only under these circumstances can the true rate of uptake be observed; otherwise, the observed reaction order will deviate from zero order, as demonstrated elsewhere (LaMotta, thesis, 1974).

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