ABSTRACT

KINETICS OF THE CONVERSION OF GLUCOSE TO

GLUCONIC ACID BY *Pseudomonas ovalis*

by

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Aerobic fermentations are usually found to proceed at a rate that is independent of dissolved oxygen activity in the fermentation medium, provided the activity is above a "critical" value which is characteristic of the organism involved. It was shown theoretically that such fermentations are controlled by chemical reaction rates, rather than oxygen absorption rates.

A critical oxygen concentration was shown to exist for the fermentation of glucose to gluconic acid by resting cells of *Pseudomonas ovalis*. The rate of production of gluconic acid in the pseudo-steady state is proportional to the rate of oxygen uptake. The rate of oxygen uptake was also measured by the rate of consumption of oxygen by the cells after the air supply was cut off. The oxygen uptake rate measured by this method was independent of the dissolved oxygen activity. It was equal to the rate of acid production just before the air supply was cut off.

According to the theory of the critical concentration, increasing the agitation rate should have no effect on the oxygen uptake rate when the dissolved oxygen level is above the critical. It was found that the rate of oxygen uptake in the gluconic acid fermentation,

measured by either of the two methods, increased when the agitation rate was increased even though the dissolved oxygen concentration was above the critical.

P. ovalis actually converts glucose to gluconolactone, which is non-enzymatically hydrolyzed to gluconic acid. The concentration of lactone in the fermentation medium increased when the agitation rate was increased. The rate of acid production was proportional to the lactone concentration so the increase in the lactone concentration with increased agitation was responsible for the increase in the oxygen uptake rate.

In one set of experiments the rate of disappearance of lactone from the broth and the rate of production of gluconic acid were measured in the absence of molecular oxygen. The rates were altered by the presence of nitrogen bubbles in the broth.

The response of the fermentation system to a sudden reduction of the pH was also evaluated. The net rate of lactone formation just after the pH was reduced was found to be higher than it had been just prior to this event.

A kinetic model of the fermentation was developed which predicts that the acid production rate in the steady state will be proportional to the lactone concentration and independent of the dissolved oxygen concentration when the latter is above the critical.

A mechanism was proposed to explain the increase in oxygen uptake as a function of increased agitation. It was proposed that the net rate of lactone formation was higher in the film around a gas bubble than in the bulk liquid. Since increasing the agitation rate

increased the gas-liquid interfacial area, the overall rate of lactone formation therefore increased with increasing agitation.

It was hypothesized that the increase in net lactone formation rate was caused by a reduced pH in the surface film when cells adsorbed at the bubble surface. Protons were attracted to the negatively charged cells, causing a reduced pH.

The effect of viscosity on the fermentation rate was investigated. Addition of sufficient hydroxyethyl ether of cellulose to reduce the oxygen absorption coefficient from air to the broth by 50 per cent had no significant effect on the gluconic acid production rate.

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in
The University of Michigan
1968

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UMR 1078

To Johanna

ACKNOWLEDGEMENTS

My appreciation and gratitude are extended to the Chairman of my Doctoral Committee, Dr. Lloyd L. Kempe. His inspiration, encouragement, and patience were invaluable, and my debt to him can never be repaid. I also thank the members of my Doctoral Committee, especially Dr. Jerome S. Schultz, for many helpful suggestions during the course of this work. My special thanks to Dr. J. L. York, who had faith in me when it was needed most.

This thesis would not have been possible without the support received from the United States Public Health Service through the Bioengineering program at the University of Michigan, and from the National Science Foundation.

Messrs. Charles Timbers, Russell Buss, and Robert Bratzler contributed materially to this thesis through their undergraduate research projects. The assistance in the laboratory of Mr. Robert Culver was also most helpful.

Many discussions with Dr. Gary F. Bennett, Dr. Stacy L. Daniels, Mr. Jens U. Pedersen, Mr. R. James Bassett, and Mr. Robert Beck were informative and appreciated.

My thanks to Mrs. J. D. Tully, who deciphered and typed my manuscript, and offered many useful suggestions.

Special appreciation is extended to my wife, Johanna, whose patience, love, and understanding cannot be adequately acknowledged.

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I. INTRODUCTION

Fermentor systems for carrying out submerged, aerobic industrial fermentations are usually sparged, agitated gas-liquid contactors. In both engineering and biological terms such systems are exceedingly complex. Mass, momentum, and heat transfer occur simultaneously with the chemical reactions of cellular metabolism. In some cases extracellular compounds such as organic acids, alcohols, and pharmaceuticals resulting from metabolism are the desired product, while in other cases maximum production of cellular material itself is desired, as in the production of food yeasts.

Mass transfer, i.e., oxygen transfer, and momentum transfer, i.e., agitation or power input, are recognized as being intimately related, and studies of aëration and agitation have been numerous and extensive (see, e.g. Ref. 3, Ch. 6 and 7). It is generally accepted that from an engineering standpoint, the ultimate objective of these studies is to provide a way of determining the level of aeration and agitation necessary to ensure maximum productivity of the desired fermentation product (32). In industrial practice the conditions for achieving the best possible productivity are almost invariably approximated by empirical methods. As discussed by Gaden (32), these methods, while grossly empirical, are sufficient to achieve acceptable productivity levels. At the same time, relatively little is known as yet about the actual mechanisms involved in supplying oxygen to fermentations.

The resistances to oxygen utilization in a submerged aerobic fermentation were enumerated some time ago by Bartholomew, Karow,

Sfat, and Wilhelm (8). These workers pointed out the existence of the following resistances:

1. Gas film resistance.
2. Gas-liquid interfacial resistance.
3. Liquid film resistance.
4. Bulk liquid resistance.
5. Liquid film resistance around the cell.
6. Internal cell resistance.

The last resistance should actually be broken up into two resistances: diffusion across the cell wall to the enzyme locus, the cytoplasmic membrane, and a resistance due to reaction at this locus.

Calderbank (15, 16) has reported an exhaustive series of experiments in which absorption coefficients were measured for a wide range of hydrodynamic conditions. It was shown that for absorption of oxygen into aqueous solutions the gas film resistance and the gas-liquid interfacial resistance are negligible compared to the liquid film resistance around the bubble for most conditions.

Tsao and Kempe (79), in presenting dimensionless correlations for the effects of agitation and aeration in fermentors, suggested that the rate limiting step for the utilization of oxygen in the gluconic acid fermentation by _Pseudomonas ovalis_ was at the cell-liquid interface. They based this suggestion on the experimental finding that increasing the agitation rate of the aerated medium increased the oxygen utilization rate of the cells. Finn (31), however, has shown that this resistance must be small compared to the absorption resistance at the liquid film around gas bubbles in the case of

yeast cells. Finn pointed out that the worst conditions for oxygen transfer from the bulk liquid in a fermentation medium to yeast cells would occur when cells were suspended in an infinite quantity of stagnant medium. Under these conditions it has been shown (46) that the Sherwood number is

$$N_{Sh} = k_m d/D_L = 2 \qquad (1)$$

Using oxygen uptake data for yeast published by Hixson and Gaden (39), taking the diameter of yeast cells as 5 microns, Finn showed that the resistance to oxygen transfer of the liquid film around the cells was smaller by about three orders of magnitude than the resistance of the film around the gas bubbles. The difference is even larger for bacteria, which are smaller than yeast. Swilley, Bryant, and Busch (75), on the other hand, have published data for BOD progressions showing that under some circumstances resistance to diffusion in the liquid phase may be limiting to the overall rate.

Resistance of the bulk liquid is not ordinarily thought to be important in bench scale fermentors so long as the liquid is being agitated. Calderbank (16), however, has shown that in large fermentors it is possible for rather large differences in dissolved oxygen concentration to occur between various points in the fermentor. Concievably, under some conditions this could also be a limiting factor.

By and large, however, it appears that for most fermentations the limiting rate process will be either transfer of oxygen from bubbles into the bulk liquid, or the resistance due to actual metabolic reactions. This, of course, can only obtain when oxygen is the

limiting substrate. It should be added, also, that almost any of the various resistances can be made controlling for a given fermentation under the proper conditions, and the conclusion just reached should be considered only as the usual condition.

i) MEASUREMENT OF PHYSICAL ABSORPTION COEFFICIENTS.

There are basically two fundamentally different ways of studying aeration and agitation in fermentor systems. One is to measure the air-to-medium absorption coefficients for a particular fermentor in the absence of cells, or in the presence of killed cells. Calderbank (16) has shown that for the absorption of slightly soluble gases such as oxygen into aqueous solutions in the absence of chemical reaction the rate of absorption is given by

$$q = d\,C/dt = k_L^o a\,(C^* - C) \qquad (2)$$

where q is the instantaneous rate of absorption in, e.g. millimoles O_2/l/minute at a particular concentration driving force $(C^* - C)$, C^* is the concentration of dissolved oxygen at the gas-liquid interface in millimoles/l, usually taken as C_s, the concentration at saturation, due to the relatively small resistance to oxygen transfer of the gas phase, and C is the instantaneous dissolved oxygen concentration in the bulk of the liquid in millimoles/l. The quantity $k_L^o a$ is the specific mass transfer coefficient for physical absorption and is in consistent units of reciprocal time. The interfacial area per unit volume of liquid, a, is difficult to measure, and usually values of $k_L^o a$ are reported in the literature. Equation (2) can be integrated, assuming the product $k_L^o a$ is constant, to give

$$\ln \left[\frac{C_s - C_2}{C_s - C_1} \right] = k_L^o a \ (t_2 - t_1) \quad (3)$$

Rearranging

$$k_L^o a = \ln \left[(C_s - C_2)/(C_s - C_1) \right] / (t_2 - t_1) \quad (4)$$

where C_1 and C_2 are the instantaneous dissolved oxygen concentrations at times t_1 and t_2 respectively. Thus if the medium in a fermentor without cells or with killed cells is first stripped of dissolved oxygen with nitrogen gas, and then air is sparged at a constant rate through the medium with a constant agitation rate, a record of dissolved oxygen concentration versus time can be obtained. The specific mass transfer coefficient at the given operating conditions can then be determined as the slope of a plot of ln $(C_s - C)$ versus t, in accordance with equation (4). This method, the so-called "gassing out" method, was devised by Wise (87) and Bartholomew *et al.* (8).

The method as outlined here depends on the validity of the assumption that $k_L^o a$ is a constant. In addition, the process of integration always has the effect of smoothing experimental data, so that $k_L^o a$ may appear to be constant when in fact it is not. This situation can be avoided by the use of Equation (2) directly in a differenced form. Equation (2) can be written in such form as

$$\frac{C_2 - C_1}{t_2 - t_1} = k_L^o a \ (C_s - \frac{C_2 + C_1}{2}) \quad (5)$$

or

$$\Delta C / \Delta t = k_L^o a \ (C_s - C_{AVG}) \quad (6)$$

As $\Delta t \rightarrow 0$, Equation (6) reduces to Equation (2). Thus instantaneous oxygen transfer coefficients can be obtained from the usual

"gassing-out" experiments. If the coefficient is constant, or nearly so, then the average $k_L^o a$ can be determined as

$$(k_L^o a)_{AVG} = \frac{1}{N} \sum_{n=1}^{N} \frac{C_{n+1} - C_n}{(t_{n+1} - t_n)\left(C_s - \frac{C_{n+1} + C_n}{2}\right)} \tag{7}$$

where N is the total number of time increments chosen.

Miller (54) has reviewed the correlations for predicting mass transfer coefficients for liquid phase controlled transfer in agitated vessels. Calderbank (15, 16) measured interfacial areas in gas-liquid contactors by a light scattering technique, and $k_L^o a$ in accordance with Equation (4) for a wide range of physical properties of the medium. For agitated tank equipment he found that k_L^o is independent of bubble size and its velocity in the liquid, and could be predicted solely from the diffusivity of the absorbed component in the absorbing liquid and the viscosity of the liquid. Interfacial areas were correlated from the power absorbed per unit liquid volume.

Yoshida and Miura (89) correlated k_L^o and a for agitated contactors from measurements of the rate of absorption of carbon dioxide into aqueous sodium hydroxide solutions. These authors found that k_L^o values for their work were slightly higher than those of Calderbank.

Wilhelm, Donohue, Valesano, and Brown (85) found that for absorption of oxygen into either water or sulfite solutions up to 65 per cent of the absorption took place in the region of the agitator and sparger.

Another method of measuring air-medium oxygen absorption coefficients which is in very common use is by the catalytic air

oxidation of aqueous sodium sulfite solutions, developed by Cooper, Fernstrom, and Miller (19). In this method oxygen is absorbed into an aqueous solution of sodium sulfite to which a small amount of catalyst, usually $CuSO_4$ or $CoCl_2$, has been added. Absorption coefficients are calculated from the rate of disappearance of the sulfite by reaction with the absorbed oxygen. The process is greatly different from the process of absorption in an actual fermentation, however. Some investigators have asserted that sulfite values indicate the maximum oxygen absorbing capacity of a given fermentation unit (32), but even the values which are obtained at constant temperature, aeration rate, and agitation rate are highly dependent on sulfite concentration and catalyst concentration. Thus Udani (80) obtained absorption rates to sulfite solutions 4 to 50 times those obtained in the absence of reaction, showing that absorption coefficients for sulfite oxidation are dependent on the reaction rate. In fact, Udani was able to fit his data fairly well with absorption models based on infinite reaction rates. Astarita (7), however, has shown that sulfite oxidation is probably better described as a very fast reaction than as infinitely fast. In any case, as will be shown below, most fermentations are characterized by oxygen utilization rates for which the absorption coefficients are unaffected by the metabolic reactions under ordinary circumstances, and k_L, the coefficient for absorption with metabolic reactions, is equal to k_L^o, the physical absorption coefficient. Sulfite oxidation measurements, although quite useful for other purposes (the method was originally proposed for comparing interfacial areas of gas-liquid contactors, see Ref. 19), must be applied

only with extreme caution to actual fermentation systems (32).

ii) MEASUREMENT OF CHEMICAL ABSORPTION RATES.

The second way of studying aeration and agitation in fermentor systems is to measure the rate of oxygen utilization during an actual fermentation. There are several ways of doing this, but they all amount to mass balances for oxygen, by either direct or indirect means. The formation of such balance equations for tank type two phase reactors has been summarized recently by Schaftlein and Russell (65). For the case of an agitated, sparged tank such as a typical batch fermentor, the assumption can usually be made that both the gas and the liquid phases are well mixed. The balance equation for oxygen in the gas phase is

$$G_o y_o - Gy - K_G aVP \left(y - C \frac{H}{P}\right) = 0 \qquad (8)$$

where G_o = inlet gas flow rate, moles/min.,

G = outlet gas flow rate, moles/min.,

y_o = inlet mole fraction of O_2,

y = outlet mole fraction of O_2,

K_G = overall gas phase mass transfer coefficient, moles/ cm^2/min./atm.,

a = interfacial area per unit liquid volume, cm^{-1},

V = volume of liquid, liters,

P = total pressure, atm.,

C = oxygen concentration in the bulk liquid, moles/l,

H = Henry's law coefficient, atm.-l/mole.

The balance equation for oxygen in the liquid phase is

$$K_G aP\left(y - C\frac{H}{P}\right) - r = \frac{dc}{dt} \tag{9}$$

where r = volumetric rate of oxygen consumption by the cells. The gas phase mass transfer coefficient K_G can be defined in terms of gas phase and liquid phase resistances:

$$\frac{1}{K_G} = \frac{H}{k_L} + \frac{1}{k_G} \tag{10}$$

As noted above, the gas phase resistance, $1/k_G$, can be neglected and K_G can be written in terms of the chemical absorption coefficient:

$$K_G = k_L / H \tag{11}$$

In addition, y can be taken as the gas concentration in equilibrium with the liquid, or $y = C_sH/P$, so Equation (9) can be written

$$k_L a\,(C_s - C) = \frac{dc}{dt} + r \tag{12}$$

Also, Equation (8) can be written

$$G_o y_o - Gy = k_L a\,(C_s - C) \tag{13}$$

These equations do not apply to continuous fermentors.

Equation (12) and Equation (13) can be combined to give

$$G_o y_o - Gy = \frac{dc}{dt} + r \tag{14}$$

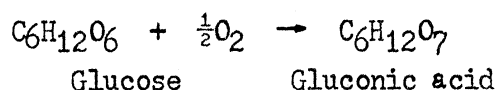
At steady state, when dC/dt is zero, then r, the oxygen consumption rate may be determined by measuring the inlet and outlet oxygen partial pressures in the gas phase (62).

In practice, oxygen absorption efficiencies, that is, the fraction of the incoming oxygen which is absorbed, are generally quite low, usually somewhat lower than one per cent (39), and accurate analyses of gas partial pressures are extremely difficult to obtain. The bulk of the present information on oxygen uptake rates has been determined rather differently. If the air supply to a fermentor or to a sample taken from the fermentor is cut off, and the fall of dissolved oxygen concentration is measured as a function of time, then from Equation (12)

$$r = -\frac{dc}{dt} \tag{15}$$

and the rate of oxygen uptake can be obtained from the slope of the C versus t curve. Usually, such plots are linear down to quite low dissolved oxygen concentrations, but if they are not, the same information can be gained from the initial slope.

Lastly, oxygen consumption rates can be estimated indirectly by determining some sort of stoichiometric ratio between oxygen uptake and some other cellular process such as growth, substrate utilization, or product formation (27, 53, 61, 64). In this connection Tsao and Kempe (79) proposed the oxidation of glucose to gluconic acid by *Pseudomonas ovalis* as a means of measuring oxygen uptake rates in a fermentor. Lockwood, Tabenkin, and Ward (50) found that *P. ovalis* oxidized glucose exclusively to gluconic acid according to the overall reaction



$$\underset{\text{Glucose}}{C_6H_{12}O_6} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Gluconic acid}}{C_6H_{12}O_7}$$

Tsao and Kempe (79), and later Bennett and Kempe (11) confirmed this, finding that 99 per cent of the glucose fermented is converted to gluconic acid by P. ovalis. Humphrey and Reilly (41) found that when the glucose concentration was low, the gluconic acid was further oxidized to 2-ketogluconic acid. These workers found, furthermore, that an intermediate gluconolactone accumulated in the broth at pH 5.8, and they actually were measuring the rate of hydrolysis of the lactone, which is not necessarily directly comparable to oxygen uptake. The hydrolysis is spontaneous, as P. ovalis does not elaborate a lactonase.

iii) EQUIVALENCE OF PHYSICAL AND CHEMICAL ABSORPTION COEFFICIENTS.

Various methods have been outlined above for measuring both physical and chemical absorption coefficients in fermentors. It has already been pointed out above that chemical absorption coefficients for sulfite oxidation, a fast chemical reaction, are a good deal higher than the corresponding physical absorption coefficients for the same equipment at the same agitation and aeration rates (80). However, it is not clear at this point what significance measured physical absorption coefficients have when compared to chemical absorption coefficients measured in actual fermentations. Astarita (7) has outlined a procedure for answering this question in a particularly clear manner. Astarita showed that gas absorption processes could be classified into several regimes, according to the relative rates of physical absorption with a maximum concentration driving force, and of chemical reaction if the absorbing liquid were saturated with the absorbed gas. There are thus three relative regimes defined by

Astarita: the slow reaction regime, the fast reaction regime, and the instantaneous reaction regime. The fast and instantaneous regimes will not be discussed here. For more information consult Astarita's text (7). Astarita proposed two parameters in the determination of which regime obtains in a given absorption process with chemical reaction. First is the "equivalent diffusion time," t_D, given by the equation

$$t_D = D_L / (k_L^o)^2 \tag{16}$$

where D_L is the diffusivity of the absorbing component, and k_L^o is the physical absorption coefficient. Note that t_D is the same as $1/s$, where s is the parameter in the Danckwerts surface renewal model of absorption. The physical significance of t_D is the average life of surface elements.

The second parameter used by Astarita is the "reaction time," defined as

$$t_r = (C_s - C_e) / r\,(C_s - C_e) \tag{17}$$

where C_s is the interface concentration of the absorbed component, and C_e is the concentration of that absorbed component at <u>chemical</u> equilibrium. The denominator $r(C_s - C_e)$ is the rate of chemical reaction at the maximum bulk liquid driving force $(C_s - C_e)$. The quantity $(C_s - C_e)$ in the denominator is the argument of r and not a multiplier.

Thus t_D is the average time available for reaction in surface elements before they are returned to and mixed with the bulk liquid, and t_r is the minimum time necessary for reaction to take place to a

significant extent. If $t_D << t_r$, that is, if

$$\frac{D_L}{(k_L^o)^2} << \frac{(C_s - C_e)}{r\ (C_s - C_e)} \tag{18}$$

then there will not be a significant amount of reaction taking place in a surface element during the life of that particular element, and the absorption coefficient will not be affected by the chemical reaction, that is

$$k_L = k_L^o \tag{19}$$

Systems for which Equation (18) is satisfied operate in the slow reaction regime. The equivalence of physical and chemical absorption coefficients in the slow reaction regime does not imply that absorption rates will be unaffected by the presence of chemical reaction, as the actual driving forces for both processes must be taken into consideration.

Astarita has further divided the slow reaction regime into two subregimes. One is the diffusional regime, for which the following must be fulfilled

$$r\ (C_s - C_e) >> k_L^o a\ (C_s - C_e) \tag{20}$$

The contemporary fulfillment of the conditions of Equation (18) and Equation (20) implies that

$$t_D << t_r = (C_s - C_e)\ /\ r\ (C_s - C_e) << 1/k_L^o a \tag{21}$$

In the diffusional regime, the reaction rate per unit interface is much larger than the absorption rate at equal driving forces, and the

actual bulk liquid concentration C is very close to the equilibrium concentration C_e.

The second subregime of the slow reaction regime is termed the kinetic regime by Astarita. In this regime

$$r \, (C_s - C_e) << k_L^o a \, (C_s - C_e) \tag{22}$$

For all reactions except zero-order, Equation (22) implies that

$$C_s - C << C - C_e \tag{23}$$

and the liquid phase is everywhere nearly saturated with the absorbing gas, i.e. $C = C_s$.

It should be clear that there are transitions from the slow reaction regime to the fast reaction regime, for which $t_D >> t_r$, as well as transitions from the diffusional to the kinetic subregimes. The latter is true for reactions of any order except zero. For true zero order reactions the transition is abrupt, and for such a reaction any absorber which operates with $C > C_e$ is operating in the kinetic regime.

Winzler (86) asserted the existence of what has come to be known as the "critical oxygen concentration." Supposedly, at bulk oxygen concentrations below this level the rate of oxygen utilization depends on the concentration, the rate usually falling hyperbolically with decreasing dissolved oxygen tension. Above the critical point the utilization rate is supposed to be independent of oxygen concentration. Many investigators have seemingly corroborated this view, and critical oxygen concentrations have been reported in the literature for a number of organisms(2, 30, 35, 43, 64, 74, 77, 86). Thus,

according to present concepts, any aerobic fermentor which operates at a dissolved oxygen concentration above the critical can be considered as carrying out a zero order reaction with respect to the absorbed component, oxygen. Most such fermentations may also be considered as largely irreversible so that $C_e \doteq 0$. Thus any fermentation for which there is a critical oxygen concentration, and which operates with a dissolved oxygen concentration above that critical concentration, may be considered as operating in the kinetic subregime of the slow reaction regime.

This can be further demonstrated by examples from the literature. Finn (31) has given a table of peak oxygen demands of a number of active cultures, which he estimated from the literature. By far the greatest demand reported was that taken from the work of Maxon and Johnson on aeration of baker's yeast. Finn pointed out that the values were a good deal higher than those ordinarily obtained in batch culture since the fermentation was continuous and cell populations were high. Maxon and Johnson reported a peak demand of 340 millimoles of O_2 per liter per hour. These workers reported a maximum value of k_La in their fermentor of 2,650 hr^{-1}, based on sulfite oxidation. This is an extremely high coefficient, and probably should be used with care. An estimate of k_L^o can be made from the data of Calderbank (16) as about 0.06 cm/sec using a value of 2×10^{-5} cm^2/sec for the diffusivity of oxygen in the liquid. The diffusion time can be estimated from these figures as

$$t_D = \frac{D_L}{(k_L^o)^2} \doteq \frac{2 \times 10^{-5}}{(6 \times 10^{-2})^2} = 5.6 \times 10^{-2} \doteq 6 \times 10^{-2} \text{ sec.}$$

From the value of 340 mmol O_2/l/hr = 9.5×10^{-2} mmol O_2/l/sec, and taking $C_s - C_e$ as 0.2 mmol O_2/l, t_r can be estimated as

$$t_r \doteq 0.2/0.095 = 2.1 \text{ sec.}$$

so

$$t_D / t_r \doteq 6 \times 10^{-2}/2.1 \doteq 3 \times 10^{-2}$$

Unfortunately, Maxon and Johnson did not report the dissolved oxygen concentrations obtained in the bulk liquid during their work, so it is impossible to tell whether the reaction rate assumed is meaningful. However, the low rates of ethanol formation obtained might indicate a finite bulk oxygen concentration. Note that use of the sulfite oxidation value and a typical interfacial area of <u>ca.</u> 1.0 cm^{-1} (15) results in an even lower ratio t_D/t_r.

Hixson and Gaden (39) have given data for baker's yeast for which the dissolved oxygen concentration was above the critical. The pertinent data are: $k_L a \doteq 15$ hr^{-1}, $r \doteq 15$ mmol O_2/l/hr. If the interfacial area is assumed to be 0.5 cm^{-1} at an agitation rate of 300 RPM, then $k_L \doteq 8.4 \times 10^{-3}$ cm/sec. and $t_D \doteq 2 \times 10^{-5}/ (8.4 \times 10^{-3})^2$ $\doteq 0.28$ sec, $t_r \doteq 0.2/(4.2 \times 10^{-3}) \doteq 48$ sec, and $t_D/t_r \doteq 6 \times 10^{-3}$. Thus it may be proposed here that according to present concepts, for all aerobic fermentations operating above the critical dissolved oxygen concentration the absorption coefficient is unaffected by physiological reactions, so that $k_L = k_L^o$.

iv) EFFECTS OF AERATION AND AGITATION ON AEROBIC FERMENTATIONS.

According to the concepts outlined above, for an aerobic fermentation operating at steady state, or at a pseudo-steady state, for

which dC/dt may be assumed to be zero, the instantaneous absorption rate should be

$$q = k_L^o a \ (C_s - C\ ) = r \tag{24}$$

The actual rate of absorption will be determined by r, which is a function of such variables as pH, temperature, specific cell activity, the number of cells, and perhaps by some other limiting substrate concentration, assuming transfer of oxygen from the liquid to the cells is not limiting. Reasons for accepting the latter for most bacterial fermentations have been outlined above.

Recently Bennett (10) and Bennett and Kempe (11) have reported experiments which suggest that the critical oxygen concentration is not well defined for the gluconic acid fermentation of *Pseudomonas ovalis*. Previously, Aiba, Hara, and Someya (2) reported that a critical oxygen concentration does exist for this organism with glucose as the substrate. They measured the rates of oxygen uptake with a dropping mercury polarograph. In the experiments of Bennett and Kempe, rates of gluconic acid production at constant pH by resting cells of *P. ovalis* increased with increasing agitation rates, even though the dissolved oxygen level was well above the critical reported by Aiba, Hara, and Someya.

The experiments of Bennett and Kempe were prompted by the earlier finding of Tsao and Kempe (79) that increasing agitation rates increased the rates of gluconic acid production for the same organism, *P. ovalis*. Tsao and Kempe, however, did not measure dissolved oxygen concentrations in their experiments, and there was a possibility that they were merely noting the effects of low oxygen tension.

Many other workers have also reported effects of agitation and aeration on fermentation rates. West and Gaden (83) found that "Increased agitation of fermentation medium, under conditions of abundant oxygen supply, improves the specific rate of growth of (baker's) yeast, but only slightly." The actual increase found in the growth rate constant was from 0.33 hr^{-1} at 250 RPM to 0.45 hr^{-1} at 1500 RPM, an increase of some 36 per cent. Given the fact that the dissolved oxygen concentration was always above the critical, this is a quite large increase compared to the zero per cent increase theoretically expected. They attributed the improvement to better bulk mixing of the mash. It should be noted that the explanation is reasonable for a zero order reaction only if poor mixing allows the dissolved oxygen to become locally depleted. This is similar to the effect predicted for zero order reactions taking place in the dispersed phase of a two phase system (see, e.g., Curl, 20). The slow rate of reaction compared to potential rates of oxygen uptake makes this unlikely, however.

In another set of experiments with baker's yeast, Siegell and Gaden (66) measured oxygen uptake by determining the difference in oxygen content between the inlet and outlet gases in a batch fermentation, in accordance with Equation (8). Uptake rates obtained in this fashion were compared with uptake rates of samples measured polarographically. It was consistently found that rates measured by oxygen balance were roughly twice those determined by the polarograph. The authors contended that the difference was due to the quiescent conditions of the polarograph sample chamber compared to

the active aeration and agitation in the fermentor. On the basis of the theory of the critical oxygen concentration this should make no difference, however.

The results of West and Gaden and of Siegell and Gaden are interesting in the light of a further finding of Bennett and Kempe (11) for the gluconic acid fermentation. In addition to the increase of acid production rate with increasing agitation rate, they found no corresponding increase in the rate of oxygen uptake as measured by the rate of fall of oxygen concentration, determined with a membrane probe, when the air supply was cut off. The difference was attributed to the fact that gas bubbles were present while acid production was being measured, whereas the probe measurements were made in the absence of air bubbles. It should be pointed out that the latter is a measurement of a transient phenomenon, but it was used as a measure of a steady-state quantity.

The results outlined so far in this section apparently contradict the theoretically expected results if the concept of a critical oxygen concentration is accepted. Bennett (10) performed experiments with the gluconic acid fermentation in which agitation and total gas flow rates were held constant, and the partial pressure of the oxygen in the gas phase was varied. In this way the bulk dissolved oxygen concentration could be varied without changing either the hydrodynamic conditions of the liquid or, presumably, the specific mass transfer coefficient or the interfacial area. Inspection of Bennett's data for these experiments reveals that, above a dissolved oxygen concentration of about 40 per cent of saturation for air, the gluconic acid

production rate was not affected by dissolved oxygen tension. This implies that a critical concentration does exist for this fermentation. However, the maximum rate at an agitation rate of 400 RPM was higher than the maximum rate at 300 RPM, although acid production rates at either agitation rate were not affected by increased bulk dissolved oxygen concentration levels.

Bennett and Kempe pointed out that Bartholomew et al. (8) suggested an alternative route for oxygen transfer in addition to the pathway mentioned above. They pointed out that cells which were in the liquid film surrounding the air bubbles were subject to a much shorter oxygen diffusion path than cells in the bulk liquid, and the cell-liquid and liquid-bubble resistances would be combined in the former. Bennett and Kempe suggested that the lowering of resistance in this fashion could increase overall oxygen uptake rates. In view of the apparent existence of a critical oxygen concentration for the gluconic acid fermentation, elimination of the cell liquid film should have no effect whatsoever.

It is interesting that Aiba, Hara, and Someya (2) have published data for experiments on the gluconic acid fermentation of P. ovalis quite similar to those of Bennett and Kempe. Aiba et al. performed their experiments in an aerated, but unagitated, column fermentor, whereas the fermentor of Bennett and Kempe was agitated and aerated. In the unagitated system, a discrepancy was also noted between acid production rates at constant pH and oxygen uptake rates measured with a polarograph, the former being as much as twice the latter. These workers also found that the rate of gluconic acid production was

independent of dissolved oxygen concentration above a bulk level of 0.7 ± 0.2 ppm. Thus the findings of Aiba, Hara, and Someya in an unagitated system directly parallel and confirm those of Bennett and Kempe in a system with agitation.

Aiba _et al._ also estimated the chemical absorption coefficient k_L and the cell-liquid transfer coefficient k_m from their data. The coefficients were found to be of roughly the same order of magnitude. These authors thus were unable to find any great difference between the resistance of the bubble-liquid film and the cell-liquid film. The quantities of importance, however, are not k_L and k_m, but rather k_La and k_ma_m, where a_m is the cell interfacial area in cm^{-1}. A value for the bubble area calculated from their data is $a \doteq 0.384\ cm^{-1}$. Typically their suspensions contained on the order of 10^9 cells per ml, and with an approximate area per cell of $3.8 \times 10^{-8}\ cm^2$, $a_m \doteq 38\ cm^{-1}$. With a Sherwood number of 2 for the cells (31), $k_m \doteq 0.37$ cm/sec, so $k_ma_m \doteq 14\ sec^{-1}$. On the other hand, k_La was typically about 50 hr^{-1}, or $1.39 \times 10^{-2}\ sec^{-1}$. Thus the cell-liquid resistance to oxygen transfer was only about 10^{-3} the bubble resistance, which is similar to the estimate of Finn for yeast cells.

Miura and Hirota (55) correctly pointed out that overall resistances were controlling. In respiration of baker's yeast they found that k_L and k_m were of the same order of magnitude, while k_c, a specific reaction rate constant defined by

$$k_ca_m\ (C_{cr} - 0) \quad = \quad k_La\ (C_s - C)$$

where C_{cr} is the critical oxygen concentration, was about two orders

of magnitude smaller than k_m or k_L. However, $k_c a_m$ and $k_L a$ were about two orders of magnitude smaller than $k_m a_m$. Overall liquid-cell resistance was therefore comparatively small in their fermentation. On the other hand it can be calculated that

$$t_D = D_L/(k_L)^2 \doteq 2 \times 10^{-5}/(0.11)^2 = 1.65 \times 10^{-3} \text{ sec}$$

$$t_r = C_{cr}/k_c a_m C_{cr} = 1/k_c am \doteq 1/(5.5 \times 10^{-2}) = 18 \text{ sec}$$

so

$$t_D \doteq 1.65 \times 10^{-3} \text{ sec} << 18 \text{ sec} \doteq t_r$$

so the reaction proceeds in the slow reaction regime of Astarita, and should be independent of $k_L a$ when $C \geq C_{cr}$.

A few investigators have reported effects of inert gas agitation in anaerobic fermentations. Stansly, Schlosser, Ananenko, and Cook (71) reported that equal yields of the antibiotic polymyxin were obtained from cultures of Bacillus polymyxa when agitated with either air or nitrogen (99.7 per cent pure). They suggested that the high surface to volume ratios obtained from passage of the gas through the broth aided the removal of metabolic gases such as carbon dioxide. Adams and Leslie (1) made a similar suggestion for the 2,3-butanediol fermentation. De Becze and Liebmann (24) found that cellular yields of compressed yeast were three to four times larger when either carbon dioxide or nitrogen was passed through the beer, compared to yields obtained when no gas was distributed. Certainly neither the removal of CO_2 nor the supply of O_2 was involved in these experiments, although removal of some other volatile component may have been involved.

Various results have been obtained when the effects of aeration and agitation on mold fermentations have been studied. Donovick (25) has reported that penicillin production can be increased by increasing power input to the agitator. Rolinson (64) however, reported that a critical oxygen concentration does exist for *Penicillium chrysogenum*. Virgilio, Marcelli, and Agrimino (82) found that high power input at a critical phase of a continuous rifamycin fermentation gave greater rifamycin yields, regardless of prior power input history. They also found that oxygen demand as measured by Warburg manometric techniques at high dilution was always the same or higher than the actual oxygen uptake as measured by analysis of the fermentation exhaust gas. Better agreement between demand and uptake rates was found at high power input.

Camposano, Chain, and Gualandi (17) found that high agitation and aeration reduced yields of kojic acid from *Aspergillus flavus*. Intense agitation produced short, highly branched mycelia. Steel and Maxon (72) found that vigorous agitation increased yields in the novobiocin fermentation.

Zetelaki and Vas (90) found that when oxygen rather than air was sparged through cultures of *Aspergillus niger*, cell yield was increased by 10 to 15 per cent, but viscosity was reduced by 50 per cent. Cell walls in oxygenated cultures seemed less rigid than cell walls from aerated cultures. Glucose oxidase activity was higher in the oxygenated culture.

Zetelaki and Vas also found that rate of growth, mycelial yield, and glucose oxidase activity increased when the agitation rate was

raised from 460 to 700 RPM. The increase in glucose oxidase activity is interesting in view of the suggestion of Rolinson (64) that since the respiratory quotient of Penicillium chrysogenum mycelia depended on aeration conditions, aeration affects not only the quantity but also the kind of respiratory enzymes synthesized.

The concept of a critical oxygen concentration is well established in the literature, and current practice is usually to assume that it does exist, and that "sufficient" aeration and agitation is being provided as long as bulk oxygen concentrations are above the critical. However, it has been shown here that a considerable body of data is being accumulated showing effects which are not explained by the theory of the critical level, and which in fact seem to contradict it.

At least some of the effects noted for mycelial suspensions could be accounted for by noting that mycelia tend to grow in clumps, and although bulk dissolved oxygen levels could be high, the concentration at the center of clumps could be zero. Thus increased agitation could break up some of these clumps and production rates thereby would be increased (8, 9). Such effects would not be expected ordinarily in bacterial or yeast fermentations, where the cells are presumably operating more or less as discrete particles. The question is clouded in the case of mycelia by the fact that considerable viscosity effects are found. In bacterial fermentations, the rheological properties of the broth do not ordinarily change drastically, except in those cases where high molecular weight compounds such as dextrans are elaborated.

v) EFFECTS OF VISCOSITY ON PHYSICAL ABSORPTION COEFFICIENTS.

Calderbank (16) has determined the effects of viscosity on physical gas absorption coefficients. It was found that $k_L^o \propto D_L^{0.84}$, whereas penetration theory predicts $k_L^o \propto D_L^{0.5}$. However, diffusivity and viscosity varied simultaneously, and it was found very approximately that

$$k_L^o \propto D_L^{\frac{1}{2}} / \mu_L^{0.266} \qquad (25)$$

Many mycelial suspensions display quite high non-Newtonian, usually pseudoplastic, viscosities, as much as 300 cp or higher Brookfield viscosity. The effect of such suspensions on diffusivity is unknown. Astarita (6) has reported that aqueous suspensions of bentonite, carboxymethylcellulose, or Carbopol, all pseudoplastic viscosity inducers, can actually increase the diffusivities of carbon dioxide and ethylene. The diffusivities were calculated from absorption rates of the gases to a laminar liquid jet. The concentrations of additive used were probably somewhat higher than necessary to achieve viscosities of 300 cp. Aiba and Sonoyama (4) have also measured the diffusivity of carbon dioxide in carboxymethylcellulose solutions in about the same general concentration range as that used by Astarita, by means of a membrane technique. They found that diffusivity decreased somewhat in such solutions. Thus the available data are conflicting but it might be assumed that the diffusivity of carbon dioxide is, to a very rough first approximation, independent of the concentration of carboxymethylcellulose or similar compounds. If it is also assumed that the effect on oxygen diffusivity is approximately

the same as the effect on the diffusivity of carbon dioxide, then an order of magnitude estimate of the effect of such viscosity additives on the physical absorption coefficient could be

$$k_L^o \propto \mu_L^{-\frac{1}{4}} \qquad (26)$$

Westerterp, van Dierendonck, and de Kraa (84) found that the interfacial area in agitated, gas-liquid contactors is proportional to viscosity, but Calderbank (15, 16) found that, in the absence of solutes which reduce coalescence rates of bubbles, the most intense agitation would not reduce bubble sizes below about 2 mm in diameter. On the other hand, even fairly low agitation rates would produce fairly small bubbles, approximately 5 mm in diameter. Thus at moderate agitation rates, viscosity additives of the type under consideration might be expected to approximately double the surface area at most. Furthermore, the relative increase in surface area between water and viscous solutions should become smaller at higher agitation speeds. Therefore, an order of magnitude estimate of the change in $k_L^o a$ upon increasing the viscosity to <u>ca.</u> 300 cp with carboxymethylcellulose derivatives would be

$$\frac{(k_L^o a)\big|\mu_L = 300\text{ cp}}{(k_L^o a)\big|\mu_L = 1\text{ cp}} \doteq \frac{2}{(300)^{\frac{1}{4}}} \doteq \frac{1}{2}$$

Also

$$(k_L^o)\big|_{\mu_L=300\text{ cp}} / (k_L^o)\big|_{\mu_L=1\text{ cp}} \doteq 1/(300)^{\frac{1}{4}} \doteq \frac{1}{4}$$

Timson and Dunn (78) measured the coefficients for the absorption of oxygen into distilled water, and into aqueous solutions of Carbopol. Carbopol is a substance with viscosity inducing properties somewhat similar to carboxymethylcellulose. Timson and Dunn did not report the concentrations of Carbopol used in their study. However, the viscosity of the most dilute Carbopol sulution they used was 670 cp, measured with a Brookfield viscometer. Inspection of their data reveals that k_L^o was reduced by less than 50 per cent for the only case actually reported. This is a much smaller change in k_L^o with viscosity than was reported by Calderbank.

Yoshida, Ikeda, Imakawa, and Miura (88) found that chemical absorption rates for sulfite oxidation were decreased about 60 per cent when the viscosity was increased with glycerol to 3.6 cp. Bennett (10) reported similar results for glycerol solutions up to 50 per cent by volume (4.78 cp). Bennett's reported sulfite oxidation rates ranged from 151 meq/l/hr with no glycerol to 27.5 meq/l/hr for 50 per cent glycerol solutions. Bennett also measured sulfite oxidation rates for sucrose solutions ranging from 151 meq/l/hr in sucrose-free sulfite solutions (0.89 cp) to 7.1 meq/l/hr for solutions containing 663 grams of sucrose per liter (11.75 cp). This is in general agreement with Solomons' (67) report that 45 per cent sucrose solutions (10 cp) reduced the sulfite oxidation rate by 84 per cent, while maximum oxygen uptake measured polarographically was reduced 92 per cent. Bennett attempted to apply a correction factor to his data for the reduced solubility of oxygen in sucrose solutions by dividing the sulfite oxidation values he obtained by the oxygen solubility, but since sulfite

oxidation values are chemical absorption rates this is not a correct procedure.

Sucrose and glycerol solutions cannot be considered as models of fermentation media or mycelial suspensions except under very special circumstances. Gas bubbles in glycerol solutions, for example, behave as rigid spheres (16), so that normal coalescence of bubbles is not obtained.

Aiba and Sonoyama (4) studied the effect of sodium carboxymethylcellulose on the physical absorption coefficients of carbon dioxide and oxygen in a wetted wall tower. They found that for operating conditions corresponding to long contact times of gas with a fluid element k_L^o was proportional to the diffusivity. For short contact times k_L^o was proportional to the square root of diffusivity. Bennett (10) gave a few values of $k_L^o a$ in a laboratory fermentor as a function of the concentration of Methocel. He also gave values of the rheological properties of both Methocel and Natrosol. Data were reported as values of the parameters in the Ostwald-deWaele model for pseudoplastics. Bennett found that the greatest drop in $k_L^o a$ occurred at Methocel concentrations where viscosity development was significant. No correlation was given.

Solomons and Perkin (68) measured oxygen transfer rates of suspensions of killed Aspergillus sp. by the gassing out method of Wise and found that increasing the apparent viscosity determined at a shear rate of 1 sec^{-1} from 1 to 1000 cp decreased the maximum oxygen uptake rate $k_L^o aC_s$ from 58 to 19 millimoles/l/hr. Solomons and Weston (69), using the data of Solomons and Perkin in a slightly

modified form, claimed that "virtually all" of the oxygen transfer took place around the impeller envelope. This may be compared with the finding of Wilhelm *et al.* (85) mentioned above that up to 65 per cent of absorption to both water and sulfite solutions takes place in the domain of the sparger and stirrer, where surface creation rates are highest.

The presence of 13.5 grams of mycelium of *Penicillium chrysogenum* per liter on a dry weight basis reduced the values of $k_L^o a C_S$ by 57 per cent according to Chain and Gualandi (18).

Brierley and Steel (12) found that the addition of either *Aspergillus niger* mycelia inhibited by azide, or shredded paper pulp in concentrations up to two per cent, reduced polarographically measured $k_L^o a$ values by about 90 per cent. Sago pellets, which are not filamentous, did not reduce $k_L^o a$ by a significant amount. These writers also reported that increased viscosity increased the bubble size and decreased the turbulence produced by the agitator. This may be compared with the claim of Westerterp *et al.* (84) that increasing viscosity increased the interfacial area. Bennett (10) also said that addition of viscosity agents increased bubble size and decreased interfacial area.

vi) EFFECTS OF VISCOSITY ON OXYGEN UPTAKE RATES IN FERMENTATIONS.

There seem to be relatively few reliable data on the effects of viscosity during actual fermentations. Bartholomew *et al.* (8) reported that in the streptomycin fermentation dissolved oxygen levels fell as mycelial mass increased, but only a portion of this was due to increasing viscosity. It was stated that in general so long as

the dissolved oxygen level was above the critical and agitation was sufficient to prevent oxygen deficiency in mycelial clumps then high viscosity should not adversely affect fermentation rates.

Phillips and Johnson (60) found that dissolved oxygen levels in cultures of Aspergillus niger fell upon the development of significant non-Newtonian flow behavior. This was also found to be the case in a Streptomyces mediterranei fermentation by Virgilio, Marcelli, and Agrimino (82).

Steel and Maxon (72) measured oxygen uptake by exhaust gas analysis for the novobiocin fermentation under conditions such that oxygen supply was limiting, i.e. increases in agitation increased oxygen uptake. They found that at a constant agitation rate there was an initial sharp drop in oxygen uptake as the viscosity was increased, but as viscosity was increased above 100 cp the oxygen uptake became independent of viscosity.

Bennett (10) increased the viscosity of the medium for the gluconic acid fermentation of Pseudomonas ovalis by adding appropriate amounts of Natrosol or Methocel. He could find no significant influence of viscosity on oxygen uptake or acid production for viscosities up to 19,000 cp. Viscosity was expressed as apparent viscosity at a shear rate of 1 sec^{-1}.

Aiba and Sonoyama (5) measured the initial rate of oxygen uptake by resting cells of Pseudomonas ovalis, Bacillus megaterium, and Saccharomyces cerevisiae as a function of viscosity. Carboxymethylcellulose was used to vary the viscosity. Uptake rates as measured by polarography were not affected by viscosity for

S. cerevisiae, rates were adversely affected for *B. megaterium*, and the rate of oxygen uptake actually increased with increasing viscosity in the case of *P. ovalis*.

vii) EFFECT OF HYDROPHYLIC SOLUTES ON $k_L^o a$.

Calderbank (15) noted that, when small amounts of any of a number of hydrophylic solutes are added to water in a gas-liquid contactor, the interfacial area is greatly increased, as previously reported by Pattle (58) and Verschoor (81). Calderbank found that the effect could not be due to lowering of interfacial tension, and instead attributed the effect to a lowering of the coalescence rate of bubbles. Thus in the usual aeration of aqueous media, there is a dynamic equilibrium between bubble formation at the impeller, and bubble coalescence as the gas rises to the surface of the medium. Calderbank found that only in the presence of such solutes could stable bubbles smaller than 2 mm be produced. Preen (63) found that, in the absence of such solutes, coalescence between bubbles about 0.5 mm in diameter and large bubbles occurred almost immediately after their formation. Zieminski, Goodwin, and Hill (91) tested the effects of a number of alcohols, carboxylic acids, esters, ketones, and commercial surface active agents on $k_L^o a$ in an aerated, but unagitated, bubble column. They found that alcohols and carboxylic acids could at least double, and in some cases triple, the value of $k_L^o a$ at concentrations as low as 6.6 ppm. The writers found that the effect was more pronounced with low efficiency aerators, which produce larger bubbles. They suggested that such substances could be used to improve the absorption effeciency of fermentors.

Zieminski and Hill (92) investigated this effect further, and found that in continuous rather than batch operation, the effect could be observed at even lower bulk solute concentrations, by continuously introducing the solute at the surface of the aerator.

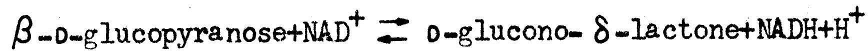
The effect is also promising to the experimenter, since it might provide a means of changing the interfacial area in a fermentor without changing the agitation rate, the air rate, or the physical properties of the broth significantly.

viii) REACTION KINETICS IN THE GLUCONIC ACID FERMENTATION.

The data of Bennett and Kempe (11), Aiba, Hara, and Someya (2), and Aiba and Sonoyama (5) on the gluconic acid fermentation by *Pseudomonas ovalis* were all collected using nitrogen-free fermentation media. In this way growth of cells was eliminated as a variable, and viable cell populations could be considered constant during the relatively short times involved for the experiments. Aiba and Sonoyama, as already pointed out, measured only initial rates of oxygen uptake, and gluconic acid production was not recorded. In the other two investigations, however, a pseudo-steady state was assumed, i.e., oxygen uptake was assumed constant, gluconic acid production was constant, and substrate (glucose) concentration was in large excess, and hence could be assumed nearly constant. Humphrey and Reilly (41) found that for *P. ovalis* an intermediate gluconolactone accumulated in significant amounts at pH 5.8. *P. ovalis* does not elaborate a gluconolactonase, and hydrolysis of the lactone to gluconic acid is spontaneous.

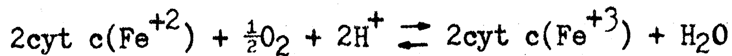
Jermyn (42) has studied both enzymatic and non-enzymatic hydrolysis of several lactones, including gluconolactone. He found that in bacterial systems containing a lactonase no lactone accumulated. At pH 7 or higher, the spontaneous hydrolysis rate was proportional to the concentration of lactone and to the hydroxide ion concentration. Below pH 7, the rate was still proportional to the lactone concentration, but the catalytic effect of ions was more complex. In any case, at constant pH, over a fairly wide range, hydrolysis of the lactone is nearly, but not completely, irreversible, and is pseudo-first order with respect to the concentration of lactone.

The gluconic acid fermentation of Pseudomonas sp. is somewhat unusual, in that it does not require phosphorylation of the primary substrate, glucose (42, 73). The first step in the reaction scheme is the direct dehydrogenation of β-D-glucopyranose to D-glucono-δ-lactone. Apparently the α form is not directly attacked (40). Eichel and Wainio (26) found that the complete system for the aerobic oxidation of glucose in mammalian liver required only glucose dehydrogenase, NAD^+, cytochrome c, and cytochrome oxidase. Participation of other cytochromes or of flavoproteins could not be detected. Reactions in cytochrome systems have been well established as reversible (29, 36, 52). Strecker and Korkes (73) were also able to demonstrate the reversibility of the lactone formation reaction. Thus, the entire reaction sequence up to the lactone hydrolysis step can be considered as reversible, and proceeds according to the scheme:

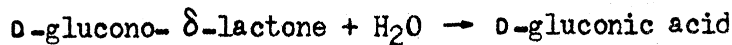


$$\beta\text{-D-glucopyranose} + NAD^{+} \rightleftharpoons \text{D-glucono-}\delta\text{-lactone} + NADH + H^{+}$$

$$NADH + H^{+} + 2\text{cyt c}(Fe^{+3}) \rightleftharpoons NAD^{+} + 2\text{cyt c}(Fe^{+2}) + 2H^{+}$$



$$2\text{cyt c}(Fe^{+2}) + \tfrac{1}{2}O_2 + 2H^{+} \rightleftharpoons 2\text{cyt c}(Fe^{+3}) + H_2O$$



$$\text{D-glucono-}\delta\text{-lactone} + H_2O \rightarrow \text{D-gluconic acid}$$

Overall:

$$\text{D-glucose} + \tfrac{1}{2}O_2 \rightarrow \text{D-gluconic acid}$$

Although the reaction sequence is simpler than many other biological oxidations, it is still so complex as to make formal treatment of the kinetics from the scheme above extremely unwieldy.

The glucose dehydrogenase which mediates the first of the reactions given above is very highly pH dependent. Brink (13) has published Michaelis constants for glucose for a glucose dehydrogenase isolated from mammalian liver. The data are given in Table I.

In the discussion of this thesis, a kinetic model is developed which predicts that, for a resting cell suspension, when sugar is present in large excess, and in the presence of an ample oxygen supply, the fermentation should reach a pseudo-steady state in which dissolved oxygen concentration, lactone concentration, and rate of acid production are all constant, provided the pH, temperature, agitation rate, and aeration rate are also constant.

ix) STATEMENT OF THE PROBLEM.

Tsao and Kempe (79) proposed a method of measuring oxygen uptake by means of a stoichiometric ratio in the gluconic acid fermentation by *Pseudomonas ovalis*. Lockwood *et al.* (50) showed, and Tsao and Kempe (79) and Bennett and Kempe (11) confirmed, that this organism

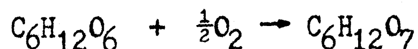
TABLE I

MICHAELIS CONSTANTS FOR A GLUCOSE DEHYDROGENASE FROM MAMMALIAN LIVER.*

pH	$K_M \times 10^2$, moles/l
6.28	34.9
6.70	8.38
7.00	3.13
7.84	3.67
8.92	32.6

*After Brink (13).

oxidizes glucose exclusively to gluconic acid by the overall reaction



$$C_6H_{12}O_6 + \frac{1}{2}O_2 \rightarrow C_6H_{12}O_7$$

In this method used by Tsao and Kempe, the cells were suspended in a nitrogen-free glucose-salts medium. In this way growth of cells did not take place, but the cells continued to respire. In such a system the rate of acid production, and hence the rate of oxygen uptake, could be calculated from the rate of alkali addition necessary to maintain a constant pH.

Tsao and Kempe found that increasing the agitation rate in their fermentor resulted in an increase in the rate of gluconic acid production, but it was not known whether the dissolved oxygen content was above the critical.

Aiba, Hara, and Someya (2) subsequently found that in an aeration column without agitation, the rate of gluconic acid production in the presence of bubbles by resting cells of P. ovalis was somewhat higher than the rate of oxygen uptake of a sample of their broth, as measured polarographically in the absence of bubbles. For their work the oxygen concentration was above the critical.

Bennett and Kempe (11) extended the work of Tsao and Kempe and found that increased agitation of their medium resulted in an increase in the gluconic acid production rate, even though the dissolved oxygen concentration was always above the critical. Concurrently, Bennett and Kempe measured oxygen depletion rates in their fermentor without taking samples. This was done by cutting off the gas supply, reducing the agitation speed to reduce surface re-aeration, and

measuring the dissolved oxygen activity with a membrane type electrode. This method, incidentally, has also been used by Korshunov, Ierusalimskii, and Skryabin (45) to determine respiration rates of cultures of the fungus *Tieghemella orchidis*. Bennett and Kempe found that the oxygen depletion rate was independent of stirring rate, and was lower than the corresponding steady state acid production rate established just prior to shutting off the oxygen supply.

Humphrey and Reilly (41) have questioned all of these results, pointing out that an intermediate gluconolactone accumulates in the broth, especially at low pH. Therefore, rates of alkali addition do not measure rates of oxygen uptake, but rather the rate of non-enzymatic hydrolysis of lactone.

Measurements of the type made by Bennett and Kempe could be put on a more substantial footing if it could be established that lactone concentration was also constant during pseudo-steady state operation. A model has been developed in the discussion section of this thesis which predicts this kind of behavior.

It has been shown earlier in this Introduction that, for reactions which display a critical oxygen concentration, oxygen uptake rates should be independent of oxygen concentration when above the critical, regardless of improvements in the overall mass transfer coefficient, $k_L^o a$. Thus, if effects similar to those of Bennett and Kempe can be demonstrated with a constant lactone concentration, then a contradiction of the critical oxygen concentration concept will be evident.

Bennett and Kempe attributed the effect they found to an elimination of the liquid-cell resistance due to adsorption of cells on bubble surfaces. Since the liquid-cell resistance is very small, its elimination should have no effect on the fermentation. However, it is still possible that the effect found by Bennett and Kempe might be due in some way to the increased bubble surface area at higher agitation rates. The chemical kinetics of the hydrolysis of the lactone can be studied in the absence of oxygen transfer effects. This can be done by establishing a steady state in a fermentation, then shutting off the air supply, and passing nitrogen gas through the fermentor. The rate of disappearance of the lactone from the fermentation broth, and the rate of production of gluconic acid, can then be measured for two cases: 1) with nitrogen passed through the head space of the fermentor, and 2) with nitrogen sparged through the broth at 1.0 VVM. Oxygen transfer effects are eliminated in both cases, since there is no molecular oxygen in the system.

The enzymatic reactions of the system are reversible; therefore, any difference between the rate of disappearance of lactone and the rate of production of gluconic acid can be attributed to a back reaction, forming glucose from the lactone. In case 1 the interfacial area will be negligibly small. In case 2, the interfacial area should be about the same as the interfacial area obtained during the steady state. Therefore, any difference in the measured rates between case 1 and case 2 can be attributed to the difference in the interfacial area.

Finally, the lack of an effect of viscosity on acid production

rates described by Bennett (10) was determined by using a different batch of cells for each viscosity level tested. It is possible to change the viscosity of the fermentation broth in the midst of a fermentation run. In this way the effect of viscosity on the fermentation rate can be determined without the uncertainties generated by using different batches of cells. When a steady state has been established in a gluconic acid fermentation at low viscosity, a higher viscosity can be relatively quickly established by adding an appropriate amount of Natrosol to the broth. This is possible because Natrosol is dispersible in cold water.

Results obtained from such an experiment could be compared with the finding of Aiba and Sonoyama (5) that high viscosity decreased the initial rate of oxygen uptake, that is, before any gluconolactone had accumulated.

II. EQUIPMENT, MATERIALS, AND METHODS

i) THE FERMENTOR SYSTEM.

All fermentations were carried out in a New Brunswick, five-liter fermentor,[1] using a working volume of two liters of liquid medium. The fermentor body was a Pyrex jar with an inside diameter of 5.75 inches and inside height of 11.75 inches. Four baffles each 0.625 inches wide were positioned at regular intervals around the inside periphery of the jar, with surfaces normal to the walls of the jar. A circular band 0.625 inches high connected the bottom ends of the baffles at a distance of 0.125 inches from the bottom of the fermentor body. The other ends of the baffles were attached to a head plate at the top of the jar.

The fermentor body rested on a rubber pad which was placed in a recess on another plate. Four studs passed up outside the fermentor jar and through holes in the head plate. The jar was held in place between the two plates by means of the studs and four wing nuts.

An agitator shaft passed through a bearing in the center of the head plate. Two flat blade turbine impellers were attached to the shaft at distances of 3.0 and 7.75 inches from the bottom of the fermentor jar. Each impeller consisted of a flat horizontal plate with four flat vertical blades attached to its edge. The blades were 0.625 by 0.625 inches, and the overall diameter of each impeller was three inches. With the impellers attached as described, the lower impeller

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[1]Model F-05, New Brunswick Scientific Co., New Brunswick, N. J.

was immersed about 2.25 inches below the surface of the liquid, and the upper impeller stirred the gas phase.

The head plate was provided with two ports for introducing gas to the fermentor. One port led through a sparger pipe to a single orifice sparger below the end of the agitator shaft, facing upwards 0.5 inches below the lower impeller. The other port led directly into the head space of the fermentor. Additional ports in the head plate were provided for sampling, for pH and oxygen electrode leads, and for the automatic addition of alkali for pH control.

The pressure of compressed air from the building supply was reduced by means of a regulator. The air passed from the regulator through a needle valve, which served as a throttle for fine control of the flow rate. The air was then bubbled through water to saturate it with water vapor. Next the air passed through the first of two five-gallon, glass carboys which served as surge dampers. From the first carboy the air was carried to a rotameter,[2] then to the second carboy, and finally either to the sparger port or to the head space port, depending on the experiment.

When inert gas was needed, pure water-pumped cylinder nitrogen was passed directly from a regulator to the rotameter, and then to the appropriate port on the fermentor. The humidifying and surge chambers were not used with nitrogen.

A glass electrode and a sleeve junction reference electrode were mounted on the baffles of the fermentor for pH control. The

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[2]Model 2F 1/4 20-5, Fischer and Porter Co., Hatboro, Pa.

sleeve junction assured an adequate flow of electrolyte in viscous solutions. The electrodes were provided with ten foot leads which passed through a port in the head plate to the pH meter.[3] The pH meter was in turn connected to a recorder-controller.[4] As the cells produced gluconic acid, the pH of the medium in the fermentor fell below the set point of the controller, which thereby activated a motor driving a Sigma pump.[5] Alkali was pumped from a 50 cc burette through rubber tubing to a length of 6 mm O.D. glass tubing, the tapered tip of which was immersed in the medium in the fermentor, just below the quiescent liquid level.

Approximately 0.5 N sodium hydroxide was used for pH control. It was standardized by titration with primary, reagent-grade, potassium acid phthalate, using phenolphthalein as the pH indicator. The alkali solution was prepared in a nearly CO_2-free condition, and was stored in polyethylene containers.

The pH of the fermentor could be controlled with a precision of better than ± 0.05 pH units by choosing a suitable pumping speed, and then standardizing the pH meter against a buffer with an external pH meter[6] at regular intervals. The recorder-controller self-standardized automatically every 15 minutes.

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[3]Model W industrial pH meter, Beckman Instruments, Inc., Fullerton, Cal.

[4]Wide-strip Dynamaster Millivolt Meter, Bristol Co., Waterbury, Conn.

[5]Finger pump, Model T-8, Sigmamotor, Inc., Middleport, N. Y.

[6]Model 76000 Expandomatic, Beckman Instruments, Inc., Fullerton, Cal.

Figure 1. Equipment used in the study of the gluconic acid fermentation.

Legend for Figure (1)

A. Indicating pH meter
B. pH recorder and controller
C. pH electrodes
D. Temperature compensator
E. Oxygen electrode
F. Modified adapter when using the No. 11098 Oxygen Electrode, Model 777 Analyzer when using the No. 39065 Oxygen Sensor
G. Millivolt recorder
H. Nitrogen tank
I. Valve
J. Air supply from building
K. Gas flow meter
L. Bath temperature controller and circulator
M. Bubbler containing distilled water
N. Surge chamber
O. Sparger
P. Baffle
Q. Sample port
R. Thermometer
S. Pulley
T. V-belt drive
U. Motor
V. Agitator shaft
W. Gas phase impeller
X. Liquid phase impeller
Y. Glass fermentor jar
Z. Head plate

a. Sigma pump
b. Driving motor for Sigma pump
c. Burette containing sodium hydroxide
d. Sodium hydroxide feed line
e. Relay
f. Constant temperature bath
g. Power source, 120 volts

The fermentor was immersed in a constant temperature water bath to a depth two or three inches greater than the liquid level in the fermentor jar. A bath water circulator and temperature controller[7] automatically maintained the bath temperature within about ±0.2 °C of the set point value. The controller was periodically checked against a mercury-in-glass thermometer that was also immersed in the bath. The thermometer had been calibrated at 0 °C and 100 °C. A temperature compensator for the pH meter was also immersed in the bath.

The agitator shaft was connected by means of an Oldham type connector to a vertical shaft mounted in a pillow block on a frame above the fermentor. Power to drive the fermentor impellers was transmitted from a pulley on the output shaft of a converted drill press by means of a V-belt to another pulley on the shaft through the pillow block. Good speed regulation was achieved due to the large excess of available torque from the drill press motor. The speed of the agitator shaft in the fermentor could be altered by changing the relative diameters of the two pulleys. Additional speed ratios could be achieved by changing the internal pulley ratio of the drill press.

ii) THE OXYGEN ELECTRODES.

Two different oxygen electrodes were used. Both were of the membrane covered, amperometric type. The important difference between the two types, for present purposes, was the relative cathode area of each. The electrode with the larger cathode area, about 1.1 square

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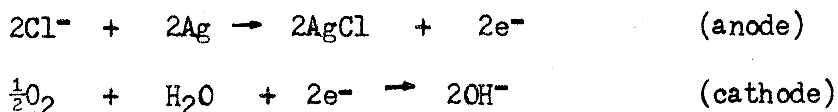
[7] Tecam Tempunit, LaPine Scientific Co., Chicago, Ill.

centimeters, was used for measurements of physical absorption coefficients.[8] This electrode was less sensitive than the smaller electrode to minor fluctuations of oxygen activity, and gave smoother reaeration curves. The larger electrode consisted of a silver anode and a platinum cathode in a glass cell. The cathode surface was flush with the open, bottom edge of the cell. A polyethylene membrane, two to four mils thick, covered the cathode and the end of the cell. The membrane was held tightly against the cathode by a plastic sleeve pushed up around the outside of the cell. Electrical continuity was provided between the cathode and the anode by a 3 per cent KCl solution.

The electrode was mounted on one of the fermentor baffles and the leads passed through the port to an adapter.[9] The adapter, originally designed for use in conjunction with a pH meter, was modified for use with a millivolt recorder.[10] The adapter circuit as modified is given schematically in Figure (2). The circuit applied a 0.6 volt potential across the electrode. As a result, the oxygen which diffused through the membrane was reduced at the cathode. The electrode reactions were



$$2Cl^- + 2Ag \rightarrow 2AgCl + 2e^- \qquad \text{(anode)}$$

$$\tfrac{1}{2}O_2 + H_2O + 2e^- \rightarrow 2OH^- \qquad \text{(cathode)}$$

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[8] No. 11098 Oxygen Electrode, Beckman Instruments, Inc., Fullerton, Cal.

[9] No. 18902 Adapter, Beckman Instruments, Inc., Fullerton, Cal.

[10] Model SR Recorder S-72180-05, E. H. Sargent and Co., Chicago, Ill.

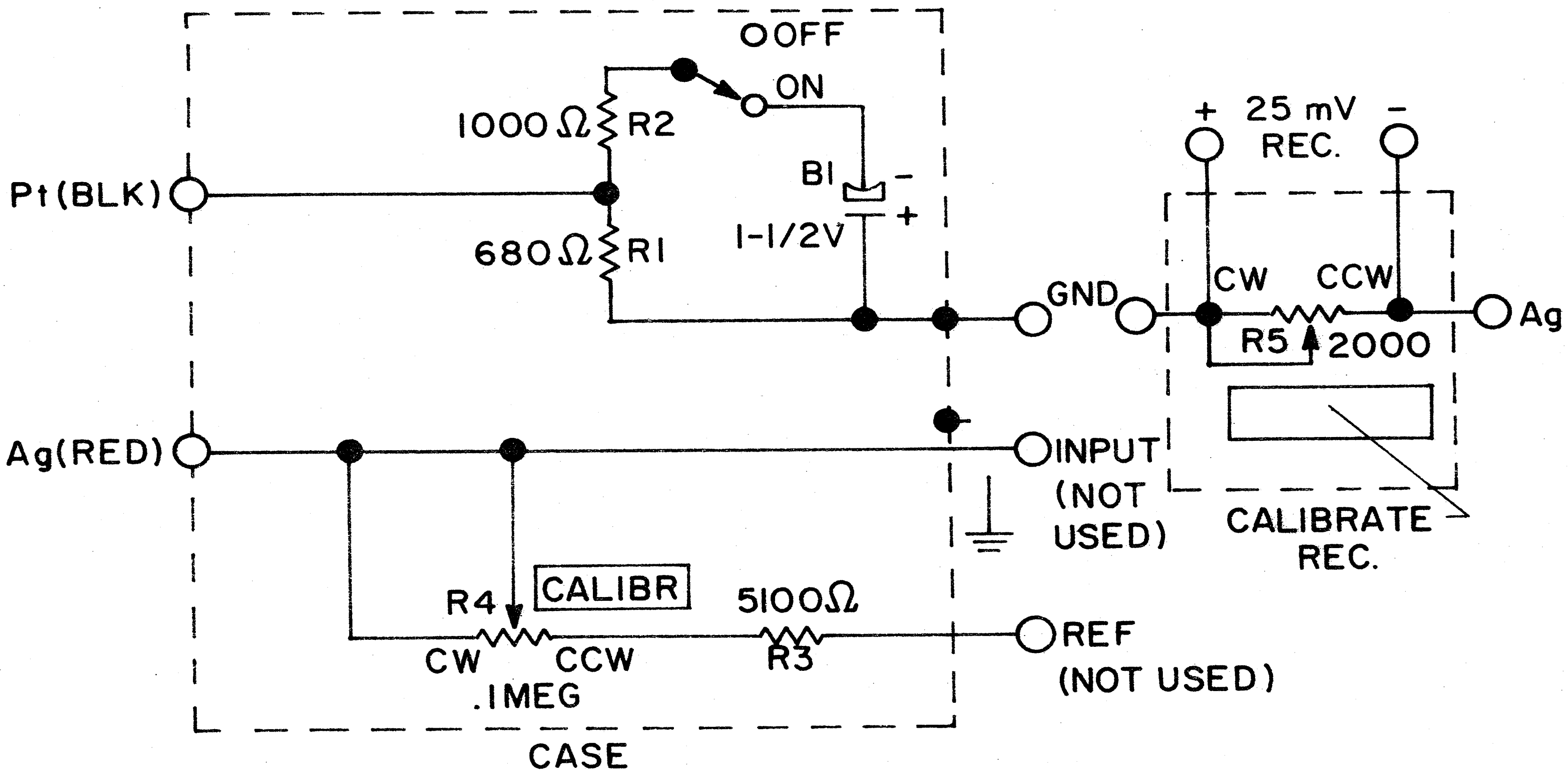

Figure 2. Oxygen electrode adapter as modified for use with a 25 mv recorder.

These electrode reactions caused a current to flow to the adapter. This adapter converted the current to a voltage that was suitable as an input to the millivolt recorder. The full-scale potential of the recorder used for this electrode was 25 millivolts.

The second model of electrode used[11] operated on the same principle as the larger electrode, but differed slightly in construction. It had a gold cathode, with an area of about 7.9×10^{-3} square centimeters, the membrane was of Teflon, and the electrolyte was a KCl gel supplied by the electrode manufacturer. The operating potential for this electrode was 0.8 volts. The electrode reactions were the same as for the larger electrode. The electrode current was indicated by an instrument[12] that also provided the 0.8 volt potential. This instrument was also provided with output terminals for connection to a 100 millivolt recorder.

The smaller electrode was used during actual fermentations and was somewhat more sensitive than the larger electrode. Automatic temperature compensation was provided by a thermistor in the electrode housing. The larger electrode was not compensated for temperature variations. This was of little concern, however, since the fermentations were carried out at constant temperature.

A third electrode, of the smaller gold cathode type, was occasionally used to measure the oxygen activity of the exhaust gas.

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[11]No. 39065 Polarographic Oxygen Sensor, Beckman Instruments, Inc., Fullerton, Cal.

[12]Model 777 Oxygen Analyzer, Beckman Instruments, Inc., Fullerton, Cal.

This electrode was inserted in one leg of a "T" type nylon tubing connector, and the "T" fitting was mounted on an exhaust port of the fermentor.

The electrodes were both standardized by the same procedure. The electrode to be calibrated was mounted in the fermentor, which was charged with the medium used for resting cell fermentations, but without carbohydrate. The fermentor was brought to 25.0 °C, and the medium was agitated and sparged with air until a steady electrode reading was obtained. The instruments were then calibrated to read full scale. Nitrogen was then sparged through the liquid until a steady residual reading was obtained. The instruments were then calibrated to read zero. The process of setting the full scale readings and the readings for zero oxygen concentration was repeated until no changes in the settings were necessary. This procedure was found to be necessary because, despite the most careful precautions, a small residual current could not be eliminated when the electrode was exposed to pure nitrogen.

Since the electrodes measured oxygen activity rather than concentration, the millivolt recorder provided a reading of dissolved oxygen in per cent of saturation. It was therefore necessary to convert the readings into concentration units. To do this, it is sufficient to know the dissolved oxygen concentration at saturation, C_s. Bennett and Kempe (11) calculated a value of C_s for their medium from the data of Solomons (67). Solomons gave data for the solubility of oxygen in glucose solutions at 37 °C. Bennett and Kempe corrected these data for temperature, and for the presence of salts and

antifoam. The medium of Bennett and Kempe was identical to that used in this study, except for the addition here of another antifoam, so their estimate of 6.8 mg/l for C_s was also used in this work.

Mueller and Boyle (56) have objected to the calculated value of C_s used by Bennett and Kempe. They contended that Bennett and Kempe calculated C_s from data on oxygen transfer from Solomons paper. They further argued that viscosity has a greater effect on oxygen transfer rates than does oxygen solubility. This is undoubtedly true for glucose solutions, but not for solutions of electrolytes. However, the question is not appropriate, since C_s was not calculated from data on transfer rates, but from oxygen solubility data at 37 °C. by assuming percentage changes in solubility at the same sugar molarity were the same at 25 °C as at 37 °C.

The misunderstanding apparently arises from a misprint in Figure (2) of Solomons' paper, where the units of the ordinate were given as mg/l/hr rather than the proper mg/l. However, it should be noted that the technique used by Mueller and Boyle to measure the change in solubility of oxygen with a change in sugar concentration is in itself subject to question. They saturated distilled water with oxygen in an air-tight reactor, noted the oxygen probe reading, then quickly added a known quantity of glucose to the reactor, and noted the new electrode reading. Since the reactor was air-tight, the solution became supersaturated with oxygen after addition of the glucose, and the probe reading consequently rose. Mueller and Boyle took the rise as a measure of the drop in oxygen solubility due to glucose. This contention is controverted by Beckman Instruments, Inc.,

in Beckman Instructions 1223-D for the use of the Model 777 Laboratory Oxygen Analyzer (April, 1967 Edition). Quoting from this booklet

> In a closed system . . . , an increase or decrease of salinity might affect the partial pressure and hence the (electrode) reading, but the reading would not be directly indicative of the change in oxygen concentration.

Such would also be the case for glucose solutions.

iii) THE ORGANISM.

The bacterial species used was a strain of Pseudomonas ovalis, NRRL B-8S, obtained from the Northern Utilization Research and Development Branch, United States Department of Agriculture, Peoria, Illinois. This strain quantitatively oxidizes glucose to gluconic acid, as reported by Lockwood, Tabenkin and Ward (50). Tsao and Kempe (79) and Bennett and Kempe (11) confirmed that there were essentially no side products developed. Humphrey and Reilly (41) pointed out that, although no side products were elaborated, the organism does not actually produce gluconic acid. Rather it produces gluconolactone, which is subsequently non-enzymatically hydrolyzed to the acid. In addition, Humphrey and Reilly found that the gluconic acid is further oxidized to 2-ketogluconic acid when the glucose concentration becomes low.

iv) MEDIA AND GENERAL CULTURING.

Medium A was used to cultivate the bacteria at 30 ^{o}C.

Medium A:

Glucose	50	grams
Yeast extract	5.0	grams

Urea	2.0	grams
KH_2PO_4	0.6	grams
$MgSO_4 \cdot 7H_2O$	0.25	grams
Distilled water	1.0	liter

Ten milliliters of the medium in screw cap test tubes were used to carry the culture from day to day. It was found that if the test tube cultures were not transferred within about 24 hours, the pH of the culture fell so low that the organisms were killed. A small amount of calcium carbonate powder was therefore added to each tube to buffer the pH at about 5.5. Although, in practice, the cultures were transferred about every two days, it was found that cultures buffered with $CaCO_3$ remained viable for at least two weeks.

One liter of Medium A was used in a two liter Erlenmeyer flask to culture the organism for a run. The flask opening was covered with a 2½ inch by 2½ inch piece of gauze cut from an 8 inch by 10 inch surgical pad.[13] The gauze was secured with a rubber band. No $CaCO_3$ was used in the flask cultures.

The stock of cultures was maintained by growing the organism in test tubes for 24 hours at 30 °C, then placing them in a freezer for storage at -20 °C. About every three months, or more often if needed, one of these frozen cultures was quickly melted, and used for innoculation of fresh tubes of medium. These cultures were then added to the cultures currently being carried.

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[13]Surgipad Combine Dressing, Johnson & Johnson, New Brunswick, N. J.

Both test tube and flask quantities of Medium A were autoclaved at 15 pounds gage pressure for 30 minutes.

Medium B was used to resuspend the cells from Erlenmeyer flask cultures.

<u>Medium B</u>:

Glucose	50	grams
K_2HPO_4	0.9	grams
KH_2PO_4	0.6	grams
Polyglycol P-2000[14]	4	drops
Silicone Antifoam B[15]	1	drop
Distilled water	2.0	liters

Polyglycol P-2000 and Silicone Antifoam B were used to control foaming. Medium B was not autoclaved, but was prepared no longer than one hour before the start of a fermentation run. Medium B contained no available nitrogen, so there was no growth of the organisms once they were added to the medium. However, respiration of such resting cultures still occurred.

v) PROCEDURE FOR A FERMENTATION RUN.

To prepare a culture for a resting cell suspension, ten ml of Medium A in a screw cap test tube were inoculated and cultured for 24 hours at 30 ± 0.5 °C. Growth took place mostly at the air-medium interface, where a rather fragile pellicle formed. This 24-hour

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[14] Dow Chemical Co., Midland, Michigan.

[15] Dow-Corning Corp., Midland, Michigan.

culture was then aseptically transferred to one liter of Medium A in a two liter Erlenmeyer flask. This medium was then incubated for 16 to 18 hours at 30 °C on a rotary shaker.[16] The flask was shaken at 180 RPM. According to Bennett (10) the cells enter the stationary phase after 12 hours, and remain in this phase up to the twenty-fourth hour. Cells incubated for 16 to 18 hours thus were in about the middle of the stationary phase of growth. The pH of the culture was normally about 4.3 at this point.

To harvest the cells, the culture was removed from the shaker and poured into a 1500 ml beaker. Three drops of Polyglycol P-2000 were added, and a propeller stirrer was placed in the culture. A pH electrode was immersed just below the surface of the medium, and the agitator was started. Sufficient 4 N sodium hydroxide was slowly added to the culture to raise the pH from 4.3 to 7.0. The cells were then aerated vigorously for 30 minutes with air blown through an Aloxite sparger. Sodium hydroxide was added intermittently to maintain the pH between 6.9 and 7.1.

The cells were next centrifuged[17] from the broth at 1060 x g for 30 minutes. The supernatant was decanted, and the centrifuge bottles were inverted for about five minutes to drain supernatant from the cell packs. The bottles were wiped dry around the inside edges, and about 50 ml of Medium B were poured into one of the bottles. The bottle was plugged with a rubber stopper, and the cells

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[16]Model VS, New Brunswick Scientific Co., New Brunswick, N. J.

[17]Model K, Size 2, International Equipment Co., Needham Heights, Mass.

were suspended in the medium by vigorous shaking. The suspension was poured successively into each of the other bottles, and the cells in each were resuspended in similar fashion. Most of the cells were thus concentrated in about 50 ml of Medium B.

There were somewhat more than 2 liters of Medium B when prepared as described above. Small portions of the cell concentrate were added to the Medium B remaining after the 50 ml was withdrawn for suspending the cells. The medium was then mixed with a magnetic stirrer. Cell concentrate was added until the turbidity of the suspension was 100 Klett units (0.20 units of optical density) on a Klett-Summerson photoelectric colorimeter[18] using a blue, 420 mμ filter. The blank used was Medium B before cells were added.

Bennett (10) studied cell populations in an identical medium, except that he used no Silicone Antifoam B. He found that a turbidity of 100 Klett units at 420 mμ corresponded to a viable cell count of about 10^7 cells per ml. Dry cell weight at this turbidity was 0.23 grams per liter, according to Bennett.

Usually about 2.01 to 2.02 liters of suspended cells were obtained after suspension as described. Exactly 2.0 liters of the suspension were used to charge the fermentor. In this way the same volume of medium of the same turbidity was used for each fermentation run. It was therefore assumed that initial cell counts were the same for all fermentations in this study.

Before the start of a run, the pH meter, the recorder-controller,

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[18]Model 900-3 Industrial Colorimeter, Klett Manufacturing Co., New York, N. Y.

and the oxygen electrode were standardized at 25 °C. For this purpose, 2.0 liters of Medium B with the glucose omitted were placed in the fermentor. The pH of a sample of this medium was measured externally. Then the pH measuring system of the fermentor was calibrated to the pH of the sample. The oxygen electrode was standardized as outlined above.

With the cells in the fermentor jar, the fermentor was assembled, and placed in the water bath, which was controlled at 25 $\pm$ 0.2 °C, and the agitation and sparging were begun. The sparging rate was 2.0 liters per minute (1.0 VVM, volume of gas per volume of liquid per minute), and the pH was controlled at 7.0 unless otherwise noted.

The pseudo-steady state was defined by three variables: 1) a constant dissolved oxygen activity, measured by the oxygen electrode, 2) a constant bulk concentration of lactone, measured by a colorimetric method described below, and 3) a constant rate of acid production, as determined by the rate of alkali addition necessary to maintain a constant pH. Readings of the sodium hydroxide level in the burette were taken every five minutes. Steady state was usually attained in about two hours.

After a pseudo-steady state had been established, the rate of oxygen utilization was measured by a transient method, using the oxygen electrode. The air was shut off, and the agitation intensity was changed from its rate during the steady state measurements to 120 RPM. During the short time (<u>ca.</u> 20-30 seconds) required to change the agitation rate, nearly all of the bubbles in the system rose to the

surface and were discharged. With the air supply cut off, the dissolved oxygen level in the fermentor fell due to its consumption by the cells. The rate of dissolved oxygen uptake by the cells was determined from the slope of the curve of dissolved oxygen versus time. A small correction factor had to be applied to compensate for reaeration of the broth from the air in the fermentor head space, as discussed by Bennett (10). Four to five minutes were usually required for the dissolved oxygen concentration to fall to a low level.

For runs in which the rate of oxygen uptake, at a steady state, was to be measured at various hydrogen ion activities, the pH was changed by the addition of either 10 N sulfuric acid or 4 N sodium hydroxide through a port in the head plate. The reagents were dispensed with a syringe. The set point of the recorder-controller was changed before the addition of acid to lower the pH, and after the addition of alkali to raise the pH.

Viscosity was changed after an initial steady state was achieved by stopping and disassembling the fermentor, inserting a propeller type agitator in the open jar to stir the broth, and carefully sifting the desired amount of Natrosol 250H[19] into the vortex of the liquid. This procedure is in accordance with the finding of Godleski and Smith (34), who found that blending in Natrosol solutions was fastest when a vortex was present, and that baffling of the vessel increased the blend time. Dispersion of the Natrosol required two to three minutes. Actual dissolution of the wetted, dispersed powder

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[19]Hercules Powder Co., Wilmington, Delaware.

took an additional 20 to 30 minutes. Viscosity progressively increased during this 20 to 30 minute period.

Natrosol 250H is a hydroxyethyl ether of cellulose. It was chosen for this study because it can be dispersed in cold water. It develops pseudoplastic viscosity in aqueous solution. Since it is nonionic, the viscosity of aqueous solutions is not significantly affected by moderate concentrations of acids, bases, and salts. Bennett (10) determined the rheological constants for aqueous solutions of Natrosol at several different concentrations. The manufacturers of Natrosol state[20] that solutions of Natrosol 250H develop a Brookfield viscosity of about 2,000 cp at 25 ^{o}C at a concentration of one per cent by weight. Further, they state that viscosity is substantially a linear function of concentration up to at least one per cent. This corresponds to a viscosity of 200 cp per gram of Natrosol per liter. Precise knowledge of viscosity characteristics was not necessary for the purposes of this study, so this figure was deemed sufficiently accurate.

vi) DETERMINATION OF LACTONE.

Lactone was measured by the hydroxamate method of Lipmann and Tuttle (48) and Hestrin (38), as modified by Lien (47). The method is based on the reaction of hydroxylamine with the internal ester of gluconolactone to form a hydroxamic acid, which reacts in turn with iron (III) in acidic solution to form a colored complex. To measure

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[20]Technical Data Bulletin VC-415-2, Hercules Powder Co., no date.

lactone concentration, a 1 ml sample from the fermentation broth was added to 2 ml of hydroxylamine reagent at 0 °C., prepared by mixing equal volumes of 4 M hydroxylamine hydrochloride and 4 N sodium hydroxide and adjusting to pH 8.0. The reaction was essentially complete in 30 seconds at 0 °C. The reaction mixture was then acidified with 1 ml of 4 N hydrochloric acid. Color was formed by the addition of 1 ml of a solution of ferric chloride, containing 100 grams per liter of $FeCl_3$ in 0.1 N HCl. The pH at this point was about 1.2. All of the reaction steps were carried out in Klett-Summerson tubes immersed in an ice bath. The contents of the tubes were thoroughly mixed after the addition of each reagent. Photometric determinations were made with the Klett-Summerson colorimeter, using a green, 540 mμ filter. Color formation was measured immediately after the addition of ferric chloride, since the color faded fairly rapidly. The blank was made up by adding the sample from the fermentor last instead of first.

A calibration curve was made by converting 1 ml samples of gluconic acid, at pH 1.5 to 2.0, quantitatively to the lactone by autoclaving at 15 pounds gage pressure for 15 minutes. The calibration curve is given in Appendix B.

The method was suitable for measuring lactone concentrations in the range 0.002 M to 0.01 M without diluting the sample.

Samples and reagents were dispensed from calibrated syringes. Although this probably resulted in some loss of accuracy, it proved necessary in order to gain sufficient precision when determining the lactone concentration of viscous samples.

vii) MEASUREMENT OF THE RATE OF LACTONE DISAPPEARANCE UNDER ANAEROBIC CONDITIONS.

For these experiments, a fermentation was conducted until a steady state was achieved. Then aeration and agitation were stopped until substantially all air bubbles had disappeared. Nitrogen gas was then passed through the head space of the fermentor, or was sparged through the broth at 1.0 VVM, which was the gassing rate used in the previous aeration. Then agitation was resumed. The lactone concentration and the rate of sodium hydroxide addition were measured every four minutes, until the lactone concentration had fallen to about 0.003 M.

Determinations of the rate of lactone disappearance were made for two cases: 1) with nitrogen passed through the head space of the fermentor, and 2) with nitrogen sparged through the broth at 1.0 VVM. These determinations were made for a series of agitation rates.

viii) MEASUREMENT OF SPECIFIC PHYSICAL ABSORPTION COEFFICIENTS.

Physical absorption coefficients were measured in two liters of Medium B with no cells present. Measurements were made at 25 °C and at various agitation rates. Coefficients were measured at an air flow rate of 1.0 VVM, and at 0.0 VVM. In the latter case the only source of oxygen was through the free surface of the liquid from air in the head space.

First, the oxygen electrode in the medium was standardized as outlined above, then the medium was stripped of oxygen by sparging with pure, water-pumped, nitrogen gas. The nitrogen supply was then cut off and agitation stopped. With the liquid quiescent, the

nitrogen in the head space was replaced with air introduced through a port on the head plate. Purging was continued until the partial pressure of oxygen in the exhaust gas was constant as measured by a second oxygen electrode attached to an exhaust port on the fermentor. Uptake of oxygen by the quiescent liquid by diffusion was negligible during this period. The agitator was then turned on, and air was either sparged through the liquid at 1.0 VVM, or it was passed through the head space of the fermentor. As oxygen was absorbed by the medium, a trace of dissolved oxygen activity as a function of time was recorded.

As discussed above, the reading of the oxygen electrode was proportional to oxygen activity, so

$$C = m(E - E_o) \tag{27}$$

where E is the electrode reading at a dissolved oxygen concentration C, E_o is the electrode reading at zero dissolved oxygen concentration, and m is a constant of proportionality.

From Equation (27)

$$dC/dt = m dE/dt$$

and

$$C_s - C = m(E_s - E_o) - m(E - E_o)$$

$$= m(E_s - E)$$

Since

$$dC/dt = k_L^o a(C_s - C)$$

then

$$dE/dt = k_L^o a(E_s - E) \tag{28}$$

Equation (28) is identical in form to Equation (2), so that $k_L^o a$ can be determined directly from records of E versus t. No knowledge of the actual values of dissolved oxygen concentration is needed, and an equation corresponding to Equation (5) can be written in terms of electrode readings as

$$\frac{E_2 - E_1}{t_2 - t_1} = k_L^o a\left(E_s - \frac{E_2 + E_1}{2}\right) \qquad (29)$$

The average specific physical absorption coefficient for any given experimental conditions between times t_1 and t_2 was thus determined from Equation (29) for a number of small, consecutive time intervals.

Physical absorption coefficients for Medium B containing varour amounts of either Natrosol 250H, or Methocel 65 HG-4000,[21] were also determined by the same method. Methocel 65 HG-4000 is a mixed ether of cellulose, with hydroxyl groups on the cellulose being substituted with methoxyl and hydroxypropoxyl groups. Methocel is nonionic. It is soluble in cold water, but it is not easily dispersed in cold water. The product must be dispersed in water at 80 to 90 °C. After dispersion, it is soluble in cold water. Methocel 65 HG-4000 produces a viscosity of 4,000 cp in aqueous solution, at 2 per cent by weight of Methocel, as measured in Ubbelohde, tube-type viscometers. Viscosity is not a linear function of concentration.

The effects of iso-amyl alcohol on specific physical absorption coefficients were also investigated. One milliliter of iso-amyl

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[21] Dow Chemical Co., Midland, Michigan.

alcohol, density 0.812 g/l, was diluted to 100 ml with distilled water. This solution contained about 8 mg of alcohol per ml. One milliliter of this solution added to 2 liters of Medium B resulted in an alcohol concentration of 4 ppm, and so on. Absorption coefficients were measured by the same method as outlined above, and results were analyzed according to Equation (29).

III. EXPERIMENTAL RESULTS

i) THE SPECIFIC PHYSICAL OXYGEN ABSORPTION COEFFICIENT, $k_L^o a$.

a) The Effect of Agitation on $k_L^o a$.

A typical curve of the oxygen electrode reading as a function of time as a cell-free broth was reaerated at 1.0 VVM is given in Figure (3). It shows that the oxygen concentration in the broth approaches saturation asymptotically, as is predicted by Equation (2). Curves similar to this one were obtained for all cases studied.

Figure (4) is a plot of the slope of Figure (3), $\Delta E/\Delta t$, versus the concentration driving force $(E_S - E)$. The straight line through the origin is the average value of $k_L^o a$ as estimated from Equation (7). Figure (5) and Table II show how $k_L^o a$ at 1.0 VVM for Medium B varies with the agitator speed. The variation is similar to that found by many other workers, with $k_L^o a$ varying exponentially with the agitator speed (3).

According to Calderbank (16), k_L^o is a function of only two variables: the diffusivity of oxygen in the medium, and the viscosity of the medium. It is independent of the hydrodynamic conditions of the liquid. Therefore, k_L^o should be constant for these runs. The variation of $k_L^o a$ is probably mainly due to changes in the interfacial area, a. Calderbank (15) also discovered that there is a lower limit to the bubble size attainable in a contactor of this type, when there is a dynamic equilibrium between liquid shear and the coalescence of bubbles. Hence, there should be an upper limit on the interfacial area which can be obtained. This may explain why

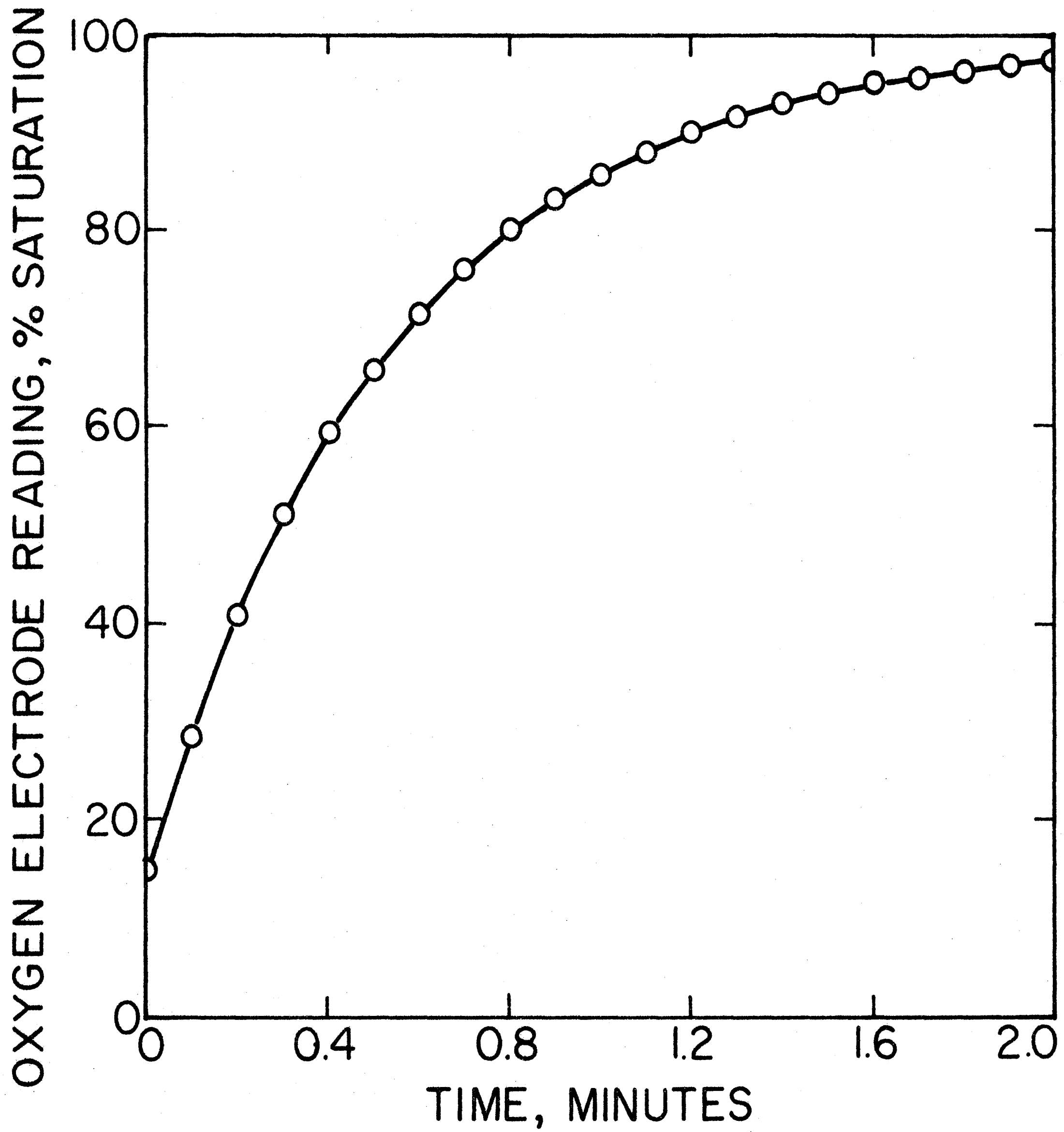


Figure 3. Reaeration curve for Medium B which had been stripped with nitrogen. Agitation rate, 434 RPM; aeration rate, 1.0 VVM.

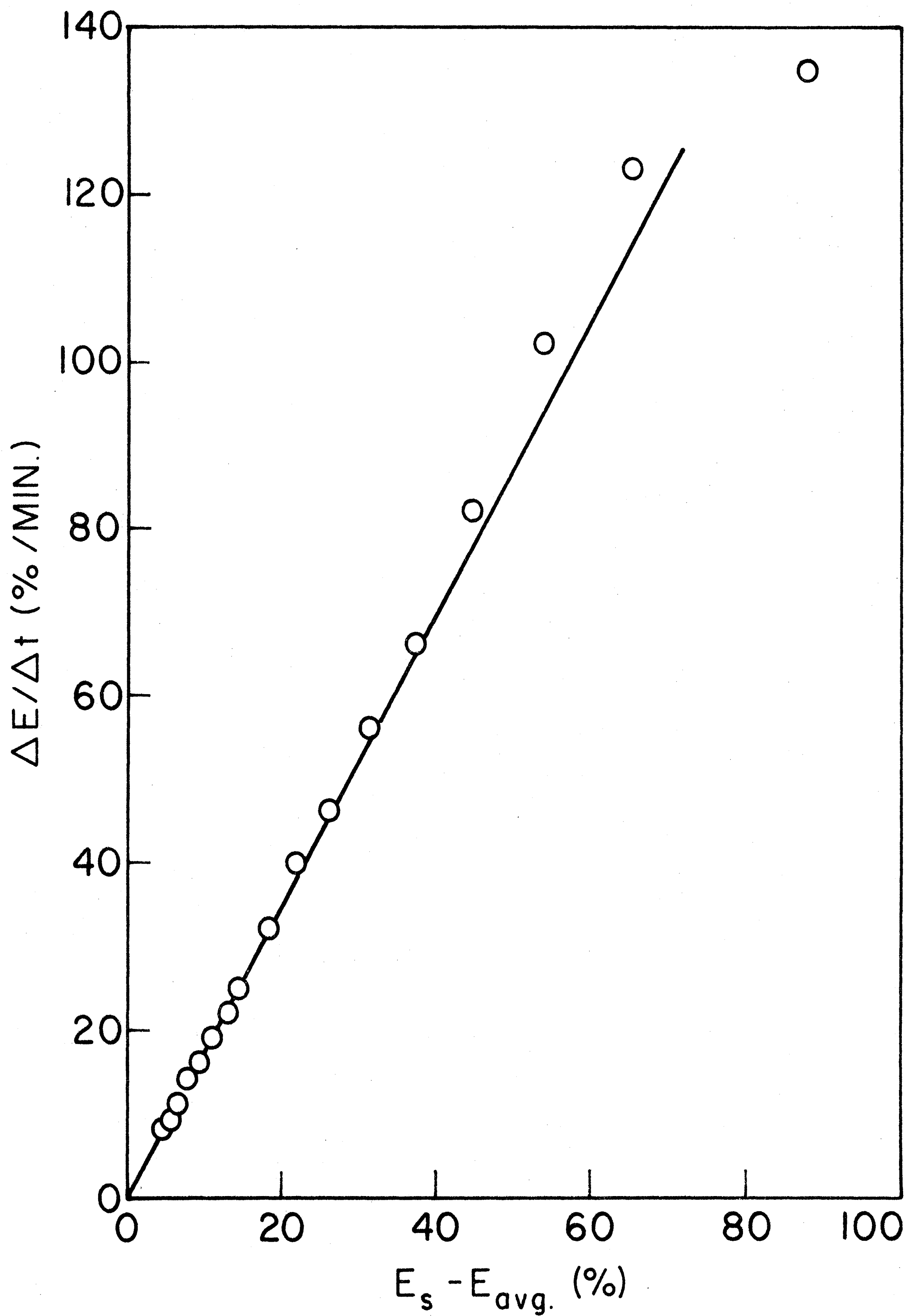


Figure 4. Rate of reaeration of Medium B which had been stripped with nitrogen. Agitation rate, 434 RPM; aeration rate, 1.0 VVM. The slope of the line is equal to $k_L^o a$.

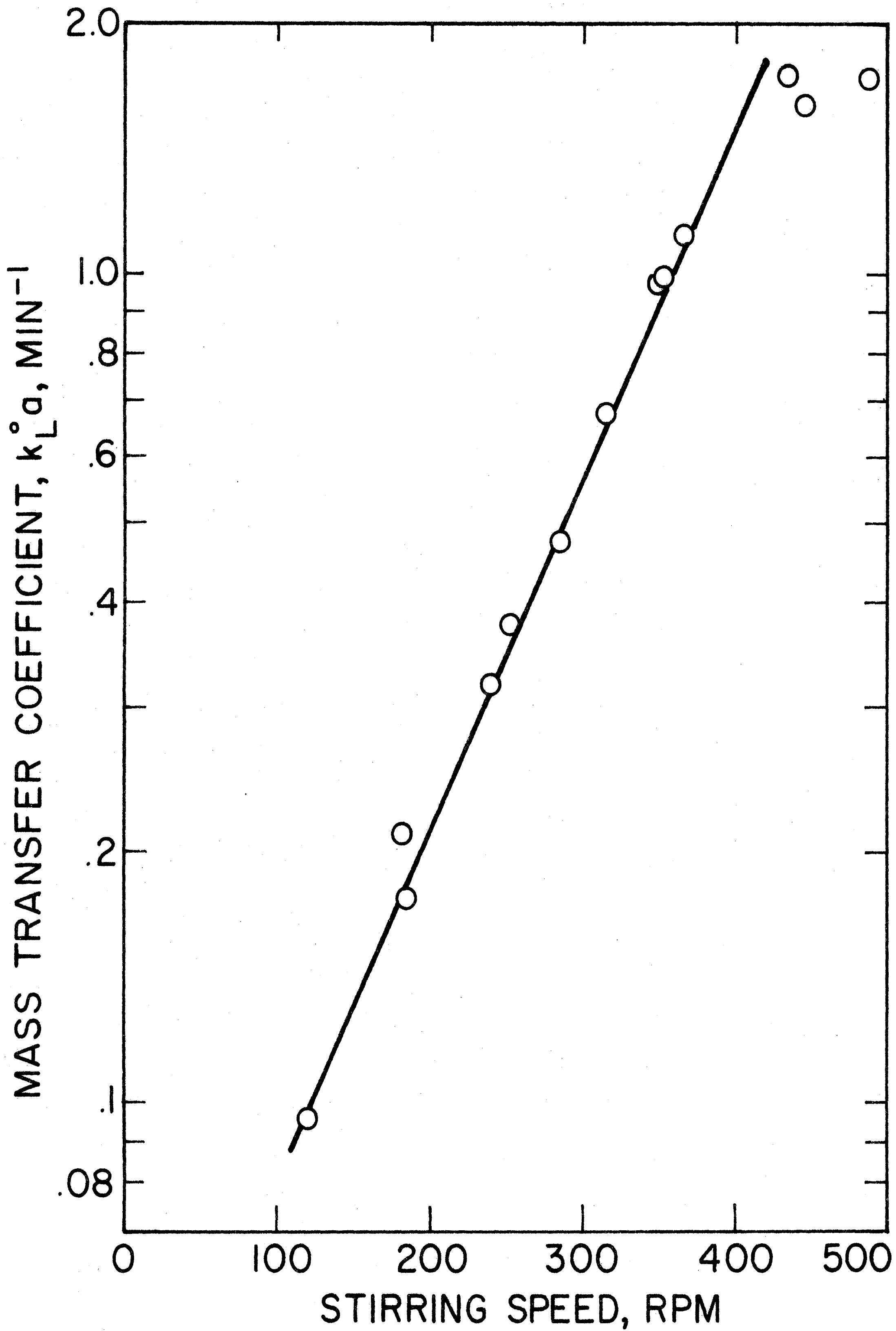


Figure 5. Oxygen transfer coefficients for Medium B as a function of agitation rate. Liquid volume, 2 liters; aeration rate, 1.0 VVM.

TABLE II

MASS TRANSFER COEFFICIENTS FOR THE ABSORPTION OF OXYGEN FROM AIR TO MEDIUM B AS A FUNCTION OF AGITATION RATE. LIQUID VOLUME, 2 LITERS, AERATION RATE 1.0 VVM, TEMPERATURE, 25 °C.

Agitation Rate RPM	$k_L^o a$ min^{-1}
121	0.096
182	0.21
185	0.18
240	0.32
252	0.38
285	0.47
316	0.68
347	0.97
353	0.99
365	1.11
434	1.75
445	1.60
485	1.72

increases in $k_L^o a$ become much smaller at high agitation rates.

b) The Effect of Viscosity on $k_L^o a$.

Values of the specific oxygen absorption coefficient were determined at various concentrations of the viscosity inducing agents Natrosol and Methocel in Medium B. Figure (6) and Figure (7) give the variation of $k_L^o a$ with agitation rate, with the concentration of Methocel and Natrosol, respectively, as parameters. Coefficients for pure Medium B with no viscosity additive are included for comparison. The data are recorded in Table III and Table IV.

It can be seen that Natrosol and Methocel profoundly affect the absorption coefficients. It was previously estimated in this thesis that increasing the viscosity from 1 to 300 cp would reduce $k_L^o a$ roughly 50 per cent. A viscosity of approximately 300 cp would be developed at a Natrosol concentration of 1.5 grams per liter. From Figure (7), it can be seen that this concentration reduced $k_L^o a$ by 41 per cent at 200 RPM, from 0.21 min^{-1} to 0.124 min^{-1}. At 400 RPM, $k_L^o a$ was reduced 55 per cent, from 1.5 min^{-1} to 0.68 min^{-1}. These results are in general agreement with those predicted in the Introduction to this thesis. The specific absorption coefficients were approximately halved at a concentration of 1.5 grams of Natrosol 250H per liter. Furthermore, the change was greater at 400 RPM than at 200 RPM. This reflects the finding of Calderbank (15) that there is a lower limit on bubble size in this type of contactor. Thus the increase in interfacial area at constant agitation rate with an increase of viscosity, which tends to offset the decrease in k_L^o, was smaller at higher agitation rates, and $k_L^o a$ was actually decreased more at higher RPM.

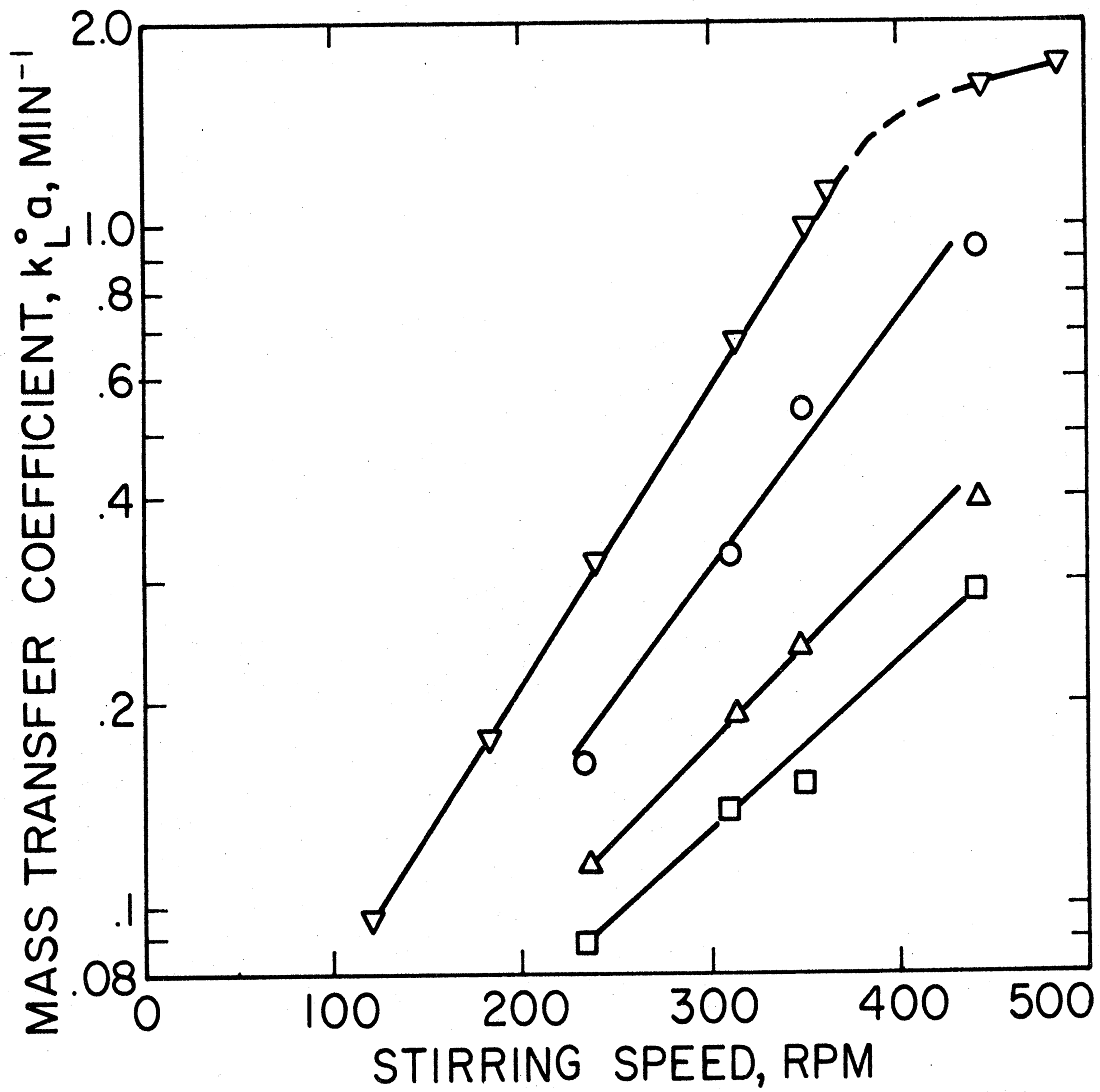


Figure 6. The effect of Methocel on oxygen transfer coefficients for Medium B. Liquid volume, 2 liters; aeration rate, 1.0 VVM. Methocel concentration: ▽, 0 g/l; ○, 2.5 g/l; △, 5.0 g/l; □, 10.0 g/l.

TABLE III

THE EFFECT OF METHOCEL 65HG-4000 ON MASS TRANSFER COEFFICIENTS FOR THE ABSORPTION OF OXYGEN FROM AIR TO MEDIUM B AS A FUNCTION OF AGITATION RATE. LIQUID VOLUME, 2 LITERS, AERATION RATE, 1.0 VVM, TEMPERATURE, 25 °C.

Methocel Concentration g/l	Agitation Rate RPM	k_La^o min^{-1}
2.5	234	0.16
2.5	311	0.33
2.5	350	0.54
2.5	440	0.93
5.0	238	0.11
5.0	314	0.19
5.0	349	0.24
5.0	441	0.39
10.0	234	0.088
10.0	310	0.14
10.0	350	0.15
10.0	440	0.29

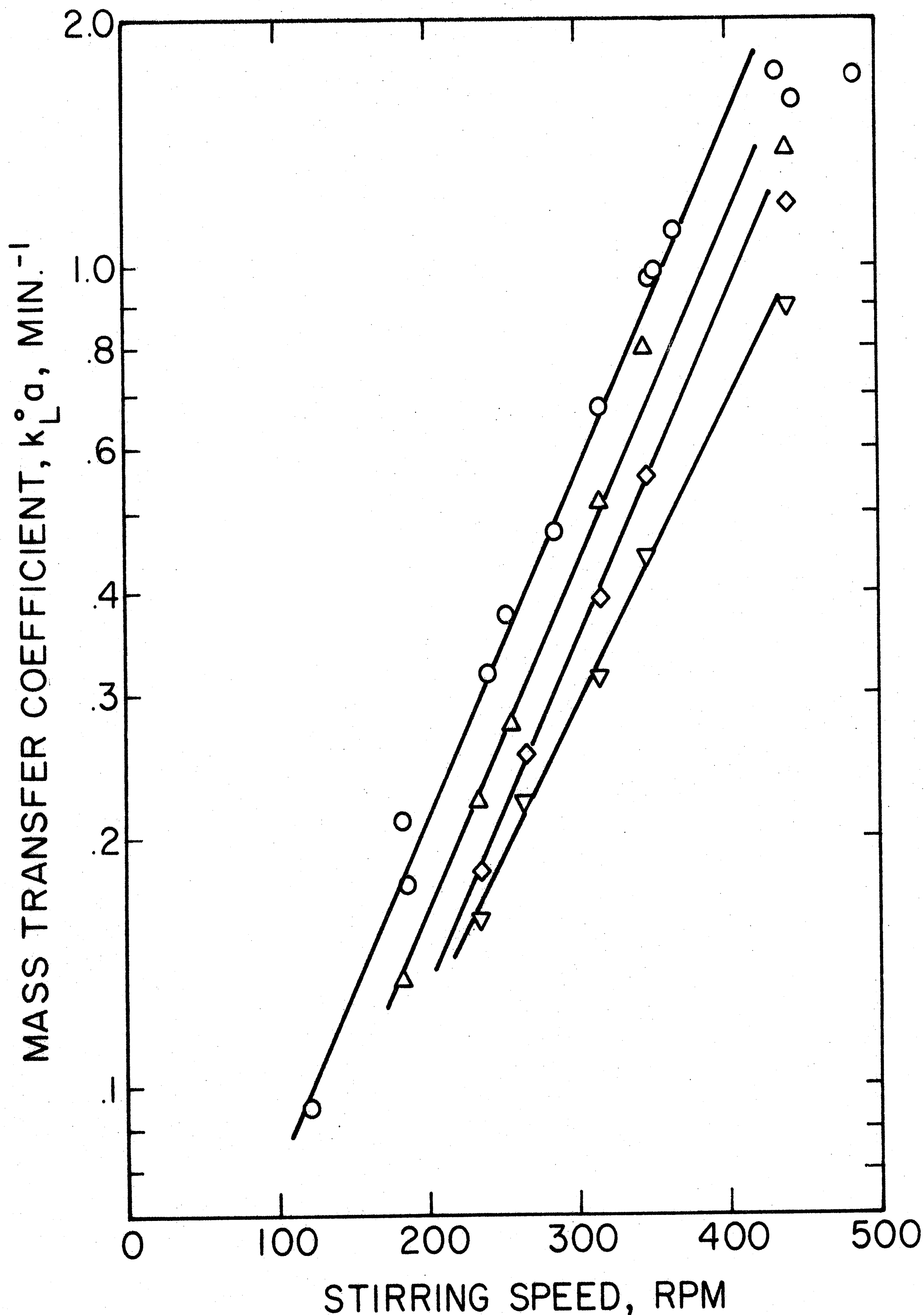


Figure 7. The effect of Natrosol on oxygen transfer coefficients for Medium B. Liquid volume, 2 liters; aeration rate, 1.0 VVM. Natrosol concentration: ○, 0 g/l; △, 0.5 g/l; ◇, 1.0 g/l; ▽, 1.5 g/l.

TABLE IV

THE EFFECT OF NATROSOL 250H ON MASS TRANSFER COEFFICIENTS FOR THE ABSORPTION OF OXYGEN FROM AIR TO MEDIUM B AS A FUNCTION OF AGITATION RATE. LIQUID VOLUME, 2 LITERS, AERATION RATE, 1.0 VVM, TEMPERATURE, 25 °C.

Natrosol Concentration g/l	Agitation Rate RPM	$k_L^o a$ min^{-1}
0.5	182	0.14
0.5	233	0.22
0.5	255	0.28
0.5	315	0.51
0.5	345	0.80
0.5	440	1.40
1.0	233	0.18
1.0	263	0.25
1.0	315	0.39
1.0	347	0.55
1.0	440	1.20
1.5	233	0.16
1.5	263	0.22
1.5	315	0.31
1.5	347	0.44
1.5	440	0.90

The prediction of the change in $k_L^o a$ with increasing viscosity was based in part on the expectation that the interfacial area would increase at higher viscosity. The interfacial area was not measured in this study, so there is no way of telling how much the interfacial area changed when the viscosity was increased. Timson and Dunn (78) reported much smaller decreases in k_L^o with increasing viscosity than Calderbank (16) did. It is possible that the increases in interfacial area with increases in viscosity were quite small. In fact, according to Brierley and Steel (12) and Bennett (10), an increase in viscosity actually results in a decrease in interfacial area. Thus, it is possible that the decrease in $k_L^o a$ was almost entirely due to a decrease in k_L^o, with the interfacial area changing very little.

c) <u>The Effect of Iso-amyl Alcohol on $k_L^o a$.</u>

Iso-amyl alcohol was added to Medium B up to a concentration of 32 ppm. No significant effect of this substance on $k_L^o a$ at 25 °C and an aeration rate of 1.0 VVM was found. When the antifoam was omitted from the formulation of Medium B, $k_L^o a$ in the presence of iso-amyl alcohol was slightly higher than $k_L^o a$ when the antifoam was present.

Zieminski and Hill (92) found that when iso-amyl alcohol was added to distilled water, the specific absorption coefficient was greatly increased at concentrations as low as 10 ppm. The effect of this substance on $k_L^o a$ for two liters of distilled water in the apparatus of this study was investigated. It was found that the coefficient increased much as reported by Zieminski and Hill. The maximum increase of $k_L^o a$ over that for distilled water occurred at a concentration of 8 ppm.

It was found, however, that when either antifoam or glucose was added to the distilled water, the effects were no longer observed. Antifoam did not alter the value of $k_L^o a$ found for either distilled water or Medium B. The results of these experiments are shown in Figure (8), and in Table V and Table VI.

It was originally anticipated that iso-amyl alcohol could be used to increase the effective interfacial area in the fermentor used for this study without altering the aeration or agitation rate, or the medium viscosity. However, it was found that the desired effect on the specific absorption coefficient $k_L^o a$ was not obtained when glucose was present in the medium. Since oxidation of glucose to gluconic acid could hardly be carried out in the absence of glucose, further investigation of this effect was abandoned.

Results of the effects of iso-amyl alcohol on the specific oxygen absorption coefficients are included here for two reasons. First, they are of interest in the light of the suggestions that have been made in the literature to use this, or similar substances, to increase absorption efficiencies in submerged fermentations (91). If the adverse effects of carbohydrate and antifoam noted here are indications of a more general inhibitory effect, then such suggestions will need to be re-evaluated.

Second, and of more importance for present purposes, is the following. The increase in $k_L^o a$ brought about by such substances as iso-amyl alcohol is usually attributed to the Marangoni effect (37, 93), that is, the alcohol prevents or decreases coalescence of bubbles in swarms, resulting in a greater effective bubble-gas interfacial area,

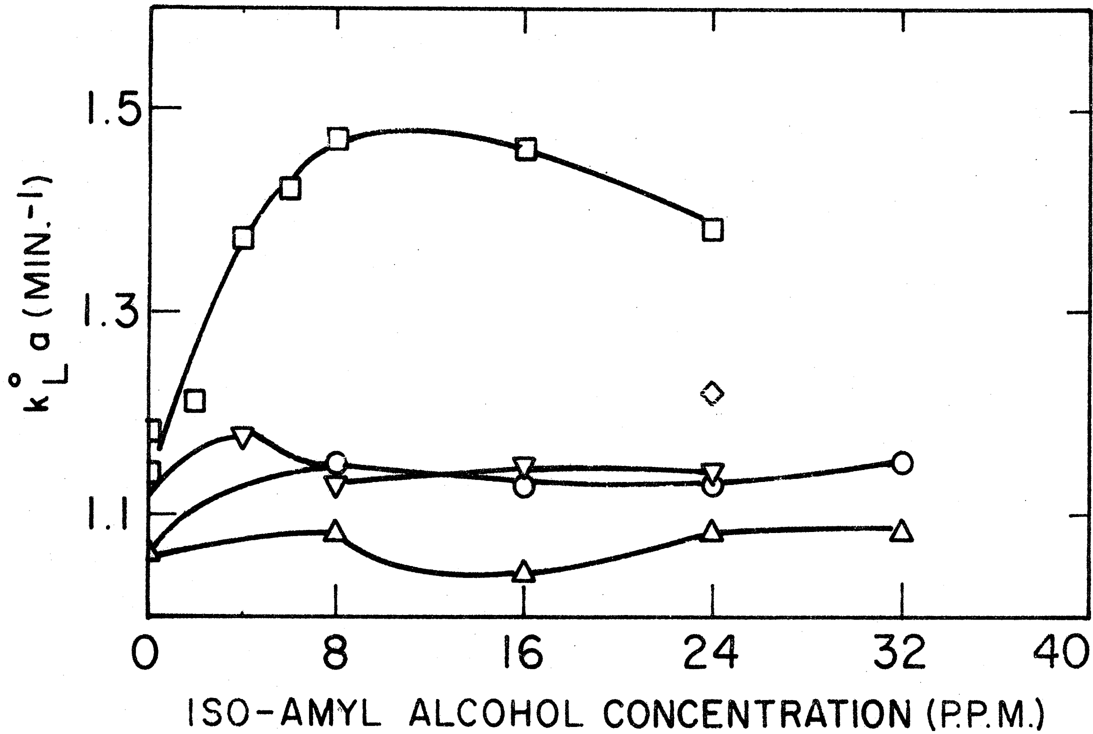


Figure 8. The effect of iso-amyl alcohol on oxygen transfer coefficients for a fermentor. Liquid volume, 2 liters; aeration rate, 1.0 VVM; agitation rate, 345 RPM.

Key:

○ Medium B without antifoam.

△ Medium B with 4 drops P-2000, 1 drop Antifoam B per 2 liters.

□ Distilled water.

◇ Distilled water with 25 grams of glucose per liter.

▽ Distilled water with 4 drops P-2000, 1 drop Antifoam B per 2 liters.

TABLE V

THE EFFECT OF ANTIFOAM ON MASS TRANSFER COEFFICIENTS FOR THE ABSORPTION OF OXYGEN FROM AIR TO MEDIUM B CONTAINING THE INDICATED AMOUNTS OF ANTIFOAM. LIQUID VOLUME, 2 LITERS; AERATION RATE, 1.0 VVM; AGITATION RATE, 345 RPM; TEMPERATURE, 25 °C.

Antifoam Added	$k_L^o a$ min^{-1}
none	1.21
none	1.26
1 drop P-2000	1.01
3 drops P-2000	1.01
4 drops P-2000, 1 drop Antifoam B	1.03

TABLE VI

THE EFFECT OF ISO-AMYL ALCOHOL ON MASS TRANSFER COEFFICIENTS FOR THE ABSORPTION OF OXYGEN FROM AIR TO DISTILLED WATER OR TO MEDIUM B WITH AND WITHOUT ANTIFOAM. LIQUID VOLUME, 2 LITERS; AERATION RATE, 1.0 VVM; AGITATION RATE, 345 RPM; TEMPERATURE, 25 °C.

Medium	Antifoam Added	Iso-amyl Alcohol Concentration ppm	$k_L^o a$ min^{-1}
Distilled	None	0	1.18
Water	"	0	1.14
"	"	2	1.21
"	"	4	1.37
"	"	6	1.42
"	"	8	1.47
"	"	16	1.46
"	"	24	1.38
Distilled Water plus 25 g/l Glucose	None	24	1.22
Distilled Water	4 drops P-2000, 1 drop Antifoam B	0	1.13
"	"	4	1.18
"	"	8	1.13
"	"	16	1.15
"	"	24	1.14
Medium B	None	0	1.06
"	"	8	1.15
"	"	16	1.13
"	"	24	1.13
"	"	32	1.15
Medium B	4 drops P-2000, 1 drop Antifoam B	0	1.06
"	"	8	1.08
"	"	16	1.04
"	"	24	1.08
"	"	32	1.08

although the rate of surface creation by shear at the impeller may be unchanged. Preen (63) reported that very small bubbles coalesce with larger bubbles almost immediately after they are created, so that these very small bubbles are never observed outside of the sparger-impeller envelope.

Since $k_L^o a$ was smaller for Medium B than for distilled water, and no increase in $k_L^o a$ for Medium B was observed upon the addition of iso-amyl alcohol, it may be concluded that coalescence rates are greater for Medium B than for distilled water, and that coalescence in Medium B is not prevented by iso-amyl alcohol.

d) Physical Absorption of Oxygen at the Free Top Surface of the Liquid.

Specific physical oxygen absorption coefficients were measured for the case in which no air was sparged through the medium, and the only source of oxygen was through the top surface of the liquid. Such a coefficient, designated herein as $(k_L^o a)_o$, might be expected to follow Equation (2), although its numerical value should be smaller than $k_L^o a$ at 1.0 VVM, due to the much smaller area available for oxygen transfer.

Figure (9) is a plot of $\Delta E/\Delta t$ versus the driving force $(E_s - E)$ for the case of surface reaeration at an agitation rate of 347 RPM. It will be recalled that for the case of aeration at 1.0 VVM a similar plot was linear (cf. Figure (4)), whereas in Figure (9) this is clearly not the case. This was found to be true at all the agitation rates studied. The coefficient at 0 VVM, which is the slope of a tangent to the curve in Figure (9), is apparently

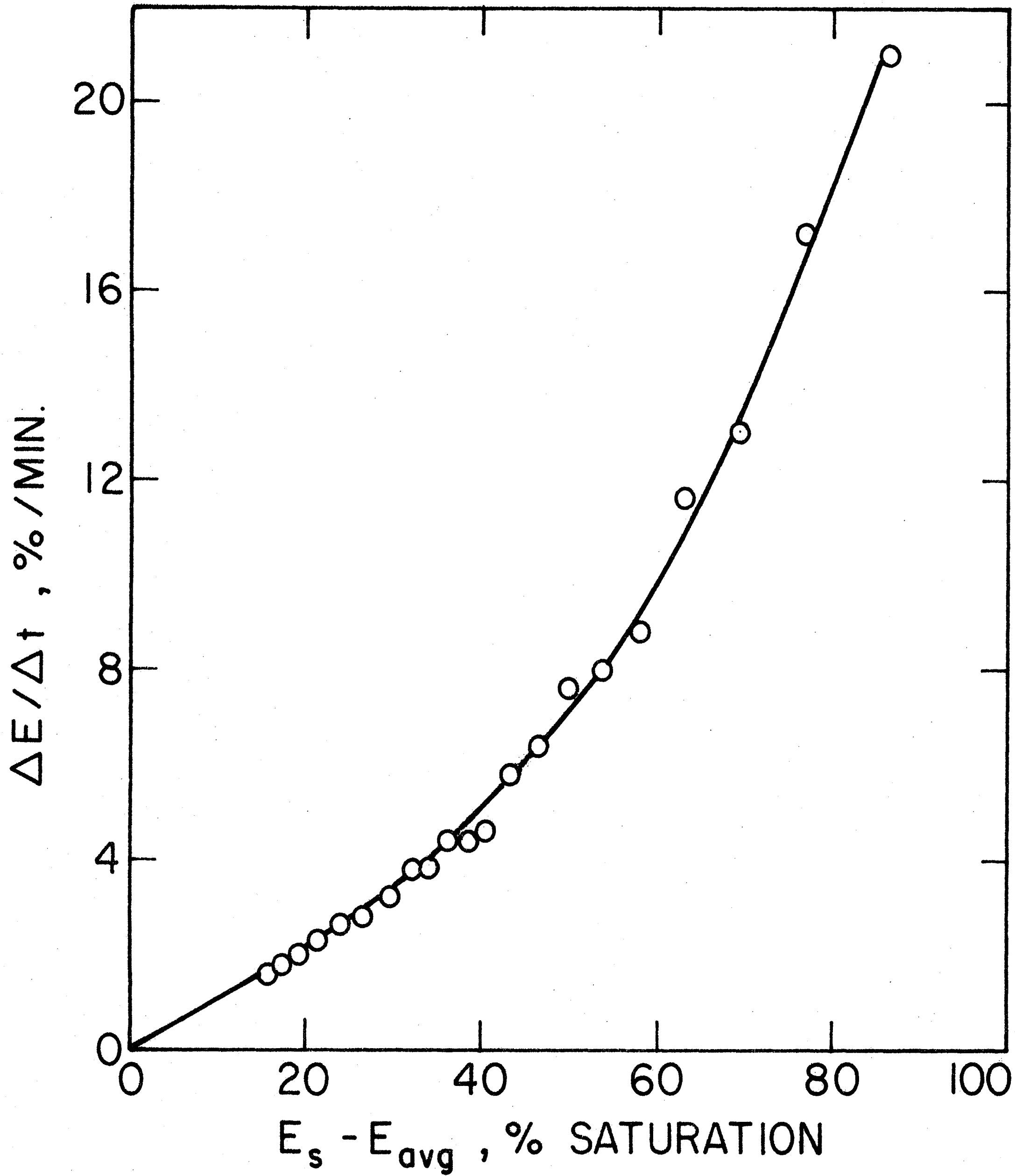


Figure 9. Rate of reaeration of Medium B which had been stripped with nitrogen. Air was supplied through the top surface of the liquid. Liquid volume, 2 liters; aeration rate, 0 VVM; agitation rate, 347 RPM. Tangents to the curve are equal to $(k_L^o a)_o$.

concentration dependent. The deviation from linearity becomes more marked at higher agitation rates.

At 1.0 VVM, when bubbles are moving through the liquid, the absorption of oxygen may be considered a penetration phenomenon. The bubbles move through the liquid, and fresh liquid is continually exposed to the bubble, with the time of contact being proportional to the velocity of the bubble relative to the liquid. Liquid turbulence and eddy diffusivity are probably rather low. However, for surface reaeration, the major means of exposing fresh liquid to the gas is by turbulence and the creation of eddies.

King (44) has recently devised a model for gas absorption incorporating a damped eddy diffusivity in the rate equations. As is usually the case, analytical solutions of the rate equations are possible only for very special conditions, but asymptotic approximations can be made. From such approximations, King predicted that for short contact times the absorption coefficient will be high. As the contact time is increased, k_L^o will fall, approaching a constant value at long contact times.

The solutions were developed for continuous, steady state operations, for which the contact time is defined by the hydrodynamic conditions of the liquid, and by the liquid residence time in the absorber. For the case at hand, the situation is somewhat different. The absorption process is transient, and the average age of surface elements can be considered as constant at a given agitation rate. The model proposed by King can nevertheless be used tentatively to explain the concentration dependence of $(k_L^o a)_o$. In King's model, short

contact times might also be considered to correspond to those conditions for which the driving force is large, while long contact times would correspond to the condition of a small driving force. Conversely, in Figure (9) the portion of the curve at a large driving force corresponds to a small contact time in King's model, whereas the portion of the curve at a low driving force corresponds to long contact times. If this were true, then $(k_L^o a)_o$ would be expected to be large at a high value of $(E_s - E)$, and then to fall to a lower, constant value at small values of $(E_s - E)$. In addition, the difference between the two limiting values should be greater when eddy diffusivity and liquid turbulence are highest, i.e., at high agitation rates. From Figure (9) it can be seen that this is the case. On the basis of this argument, it is postulated that bubble absorption is a simple penetration phenomenon, whereas surface reaeration is more adequately described by a model employing a damped eddy diffusivity, as in the model of King (44).

ii) STUDIES ON THE GLUCONIC ACID FERMENTATION OF *Pseudomonas ovalis*.

a) The Effect of Agitation on Oxygen Utilization Rates.

The effect of agitation on the gluconic acid fermentation of *P. ovalis* at steady state is shown in Figure (10) and in Table VII. The criteria of a pseudo-steady state were those outlined in the section "Procedure for a Fermentation Run": a constant dissolved oxygen activity, a constant bulk lactone concentration, and a constant rate of acid production. When these three criteria were satisfied at a particular agitation rate, the oxygen supply was cut off, and the

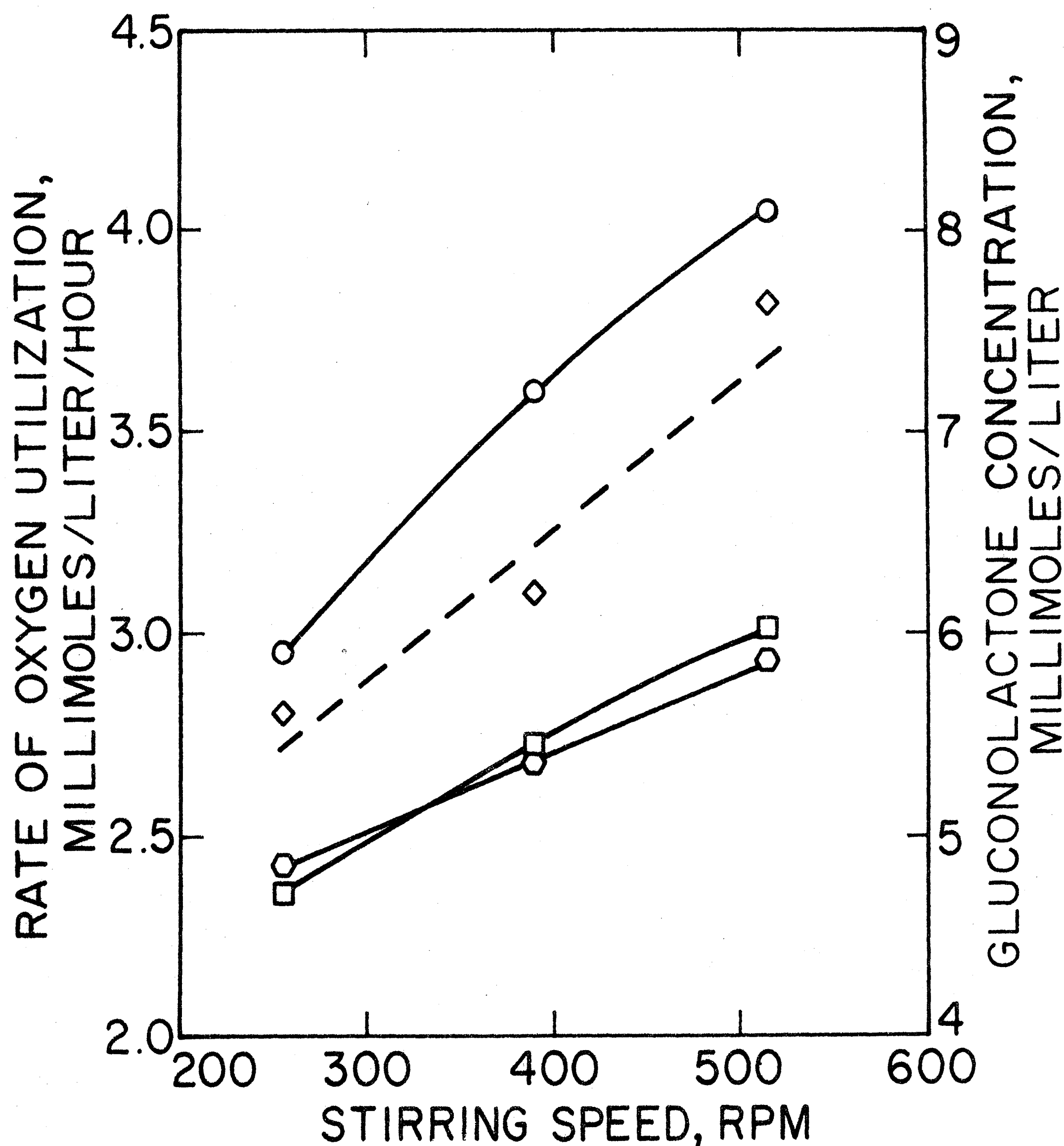


Figure 10. The effect of the agitation rate on the rate of oxygen utilization and the gluconolactone concentration in the gluconic acid fermentation by resting cells of <u>Pseudomonas ovalis</u> in 2 liters of Medium B at pH 7.0, 25 °C, an aeration rate of 1.0 VVM, and cell concentration of 100 Klett units.

Key:

□ Oxygen uptake rate measured by acid production with aeration.

◇ Oxygen uptake rate measured by acid production just after aeration was stopped.

⬡ Oxygen uptake rate measured by dissolved oxygen depletion with stopped aeration.

○ Steady state lactone concentration.

TABLE VII

THE EFFECT OF THE AGITATION RATE ON THE RATE OF OXYGEN UTILIZATION AND THE STEADY STATE GLUCONOLACTONE CONCENTRATION IN THE FERMENTATION OF GLUCOSE TO GLUCONIC ACID BY RESTING CELLS OF *Pseudomonas ovalis* IN MEDIUM B. LIQUID VOLUME, 2 LITERS; AERATION RATE, 1.0 VVM; pH, 7.0; TEMPERATURE, 25 ^oC; CELL CONCENTRATION, 100 KLETT UNITS.

	Agitation Rate, RPM		
	255	390	515
Oxygen Uptake Rate Measured by Acid Production with Aeration, millimoles/liter/hour	2.35	2.72	3.01
Oxygen Uptake Rate Measured by Acid Production Just After Aeration was Stopped, millimoles/liter/hour	2.79	3.09	3.82
Oxygen Uptake Rate Measured by Dissolved Oxygen Depletion with Stopped Aeration, millimoles/liter/hour	2.42	2.68	2.93
Steady State Lactone Concentration, millimoles/liter	5.9	7.2	8.1

rate of depletion of oxygen was recorded. The rate of alkali addition was also noted during the transient portion of the experiment. The entire process was then repeated at two additional agitation rates. For the experiment shown in Figure (10), the dissolved oxygen levels were approximately 46 per cent of saturation at 255 RPM, 86 per cent at 390 RPM, and 93 per cent at 515 RPM. These values are all well above the critical level of about 16 per cent determined by Bennett (10).

Four main points may be noted. The first is that four of the measured quantities increased with increases in agitation rates. These are the steady state acid production rate with aeration, the acid production rate immediately after aeration was stopped, the oxygen consumption rate measured by oxygen depletion with stopped aeration, and the steady state lactone concentration with aeration. Thus, one of the findings of Bennett and Kempe (11) is confirmed: the rate of production of gluconic acid, and therefore the rate of oxygen consumption, increased with increased agitation, even though the dissolved oxygen level was well above the critical.

Second, the rate of oxygen consumption does not depend on the dissolved oxygen concentration, above roughly 25 per cent of saturation. At any given agitation rate the curves of dissolved oxygen versus time after the air was cut off were more or less linear, above about 25 per cent of saturation. A correction factor for surface reaeration was applied to these curves. The slopes of the dissolved oxygen depletion curves were dependent upon the agitation rate just prior to cutting off the air.

Third, the rate of oxygen consumption measured by steady state acid production and the rate measured by oxygen depletion with stopped aeration were nearly the same. This is contrary to the finding of Bennett and Kempe (11), who found that the rate measured with stopped aeration was independent of the agitation rate just prior to cutting off the air supply. The probable explanation for this is that Bennett and Kempe, unaware of the accumulation of gluconolactone in the broth, did not allow enough time to elapse between measurements for the lactone concentration to change significantly. It is important to point out in this context that Bennett and Kempe measured acid production rates with one batch of cells, and oxygen depletion rates with another batch.

Fourth, the rate of acid production just after aeration was stopped was considerably higher than the steady state and transient rates of oxygen consumption. It is reasonable to assume that the lactone concentration during this interval was nearly constant. In fact, the oxygen depletion curves were obtained in about four minutes, and the lactone concentrations for the two higher agitation rates, measured about ten minutes after the air flow was stopped, showed that the lactone concentration fell less than ten per cent during this ten minute interval.

b) The Effect of Viscosity on Acid Production Rates.

To determine the effect of viscosity on the rate of production of gluconic acid, a steady state was first achieved without viscosity additive, then a small amount of cold-water-dispersible Natrosol 250H was added to the broth, and the effects on the fermentation were

noted. Figure (11) shows the results from a typical run. After the Natrosol was added, there was a gradual drop in oxygen tension to a new steady value. This is indicative of the time necessary for the dispersed Natrosol to dissolve, and for its viscosity effect to develop. There was no significant change in either the lactone level in the broth or in the rate of acid production after this period. If any change at all can be observed, it is a very slight increase in the rate. Thus, this substance has no significant effect on acid production in this fermentation, even though the mass transfer coefficients were drastically lowered by Natrosol in cell-free systems.

From Figure (7) it can be estimated that the addition of one gram of Natrosol per liter will reduce $k_L^o a$ from 0.45 min^{-1} to 0.28 min^{-1} at 278 RPM. From Figure (11) it can be seen that the dissolved oxygen concentration changed from 60 per cent of saturation (0.255 meq/l) to 30 per cent of saturation (0.128 meq/l) upon the addition of one gram of Natrosol per liter. Thus

$$q = k_L^o a \ (C_s - C)/_{\text{No Natrosol}} = 0.45(60)(\frac{6.8}{16})(1.0 - 0.6) = 4.6 \text{ meq/l/hr.}$$

and

$$q = k_L^o a \ (C_s - C)/_{\text{1g.Natrosol/L}} = 0.28(60)(\frac{6.8}{16})(1.0 - 0.3) = 5.0 \text{ meq/l/hr.}$$

These figures can be compared with the experimentally determined rate of acid formation of approximately 4.5 meq/l/hr. This is a further indication that the rate of oxygen uptake was unchanged by increased viscosity. The figures are somewhat uncertain since the Natrosol

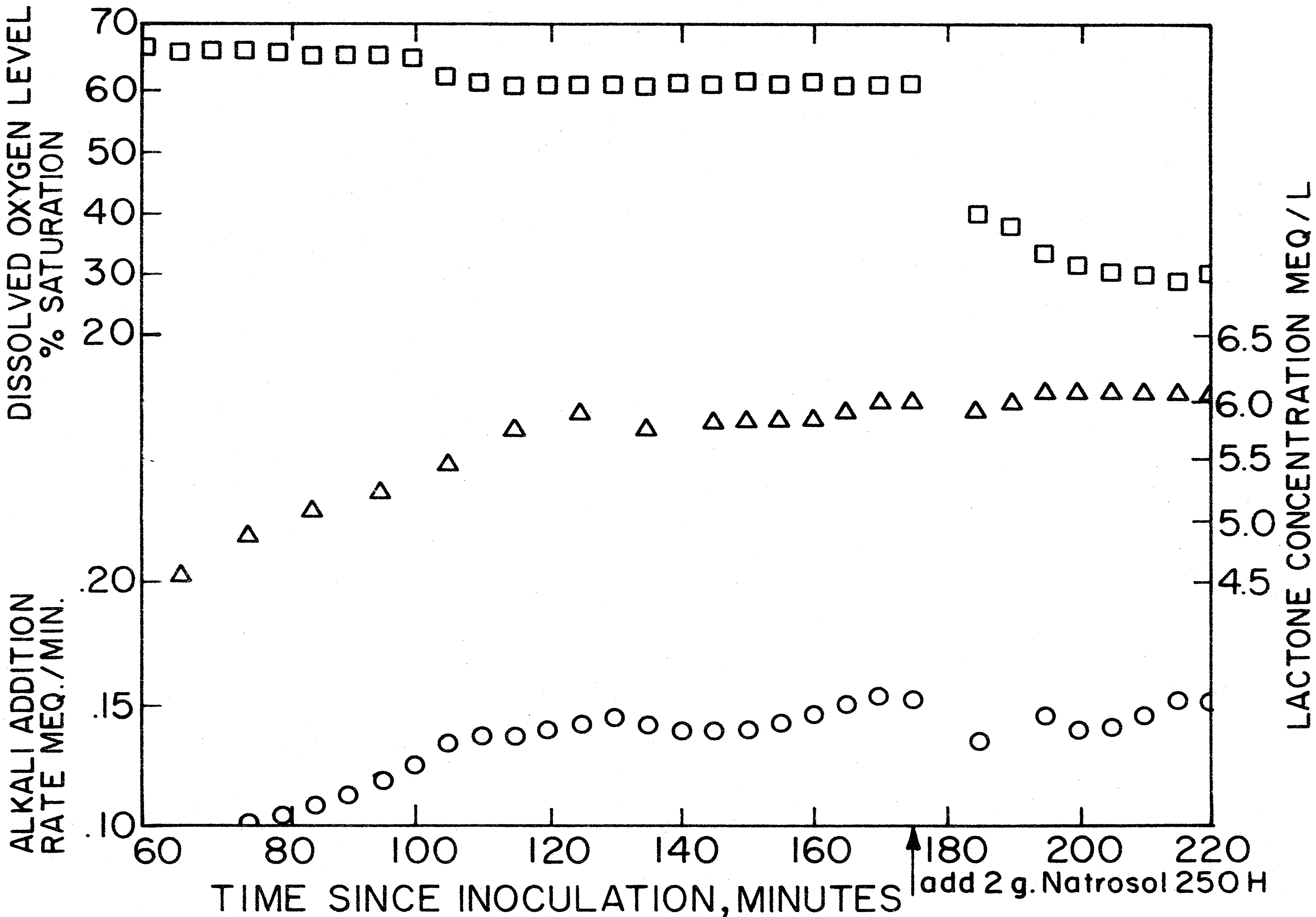


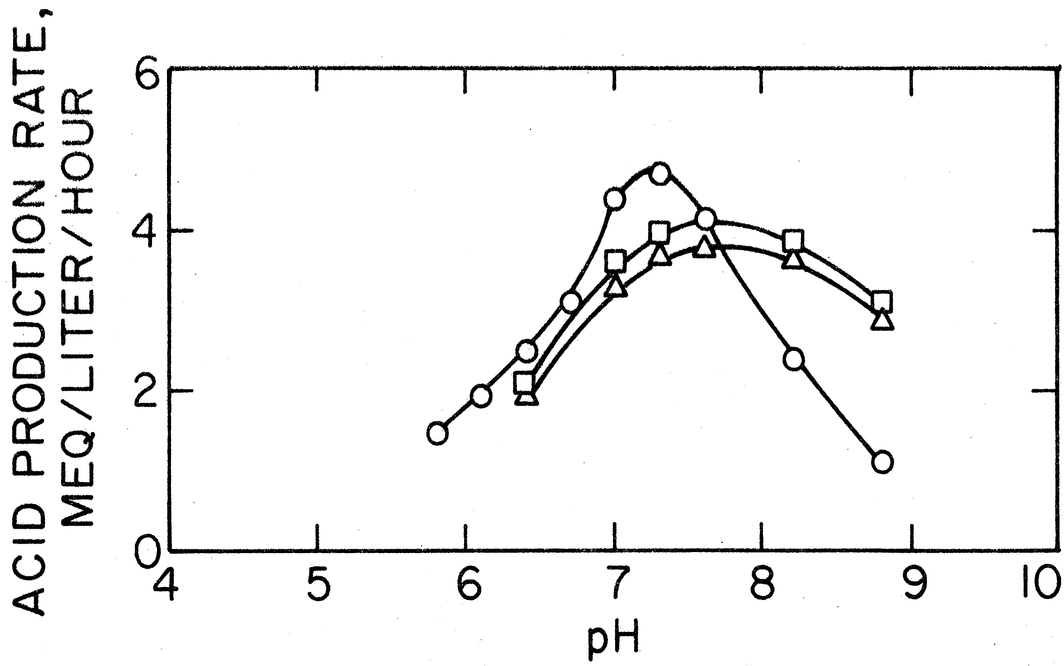
Figure 11. The effect of Natrosol on the gluconic acid fermentation by resting cells of Pseudomonas ovalis in 2 liters of Medium B. Aeration rate, 1.0 VVM; agitation rate, 278 RPM; pH 7.0; temperature, 25 °C; cell concentration, 100 Klett units.

must be measured extremely accurately in order to obtain reproducible viscosities. This experiment confirms the finding of Bennett (10), that the rate of acid production is independent of the viscosity of the medium.

c) The Effect of pH on the Gluconic Acid Fermentation.

Bennett (10) offered data concerning the effects of pH on the rate of gluconic acid production by *P. ovalis*. The data showed a maximum rate of acid production at pH 7.35. Bennett, however, did not report the changes in steady state lactone concentration with changes in pH. Experiments are reported here in which both the acid production rate and the lactone concentration at steady state were determined as a function of pH. Three runs were made: one run in which the cells were incubated for 16 hours and then centrifuged and resuspended, and two runs in which the incubation period was 20 hours. The results are presented in Figure (12) and in Table VIII. The optimum pH for acid production was sharply defined for the 16 hour culture. For the 20 hour culture, the optimum pH was not as well defined. The maximum rate was somewhat greater for the 16 hour culture than it was for the 20 hour culture. The optimum pH was 7.3 for the 16 hour culture, and about 7.5 for the older culture.

These facts are also reflected in the steady state lactone concentrations found in these runs. The lactone concentrations were relatively high at lower pH values, falling to rather low values as the pH was increased. The curves are more or less sigmoid, and the inflection points occur at or near the pH values for which acid production was maximal. The difference between the lactone



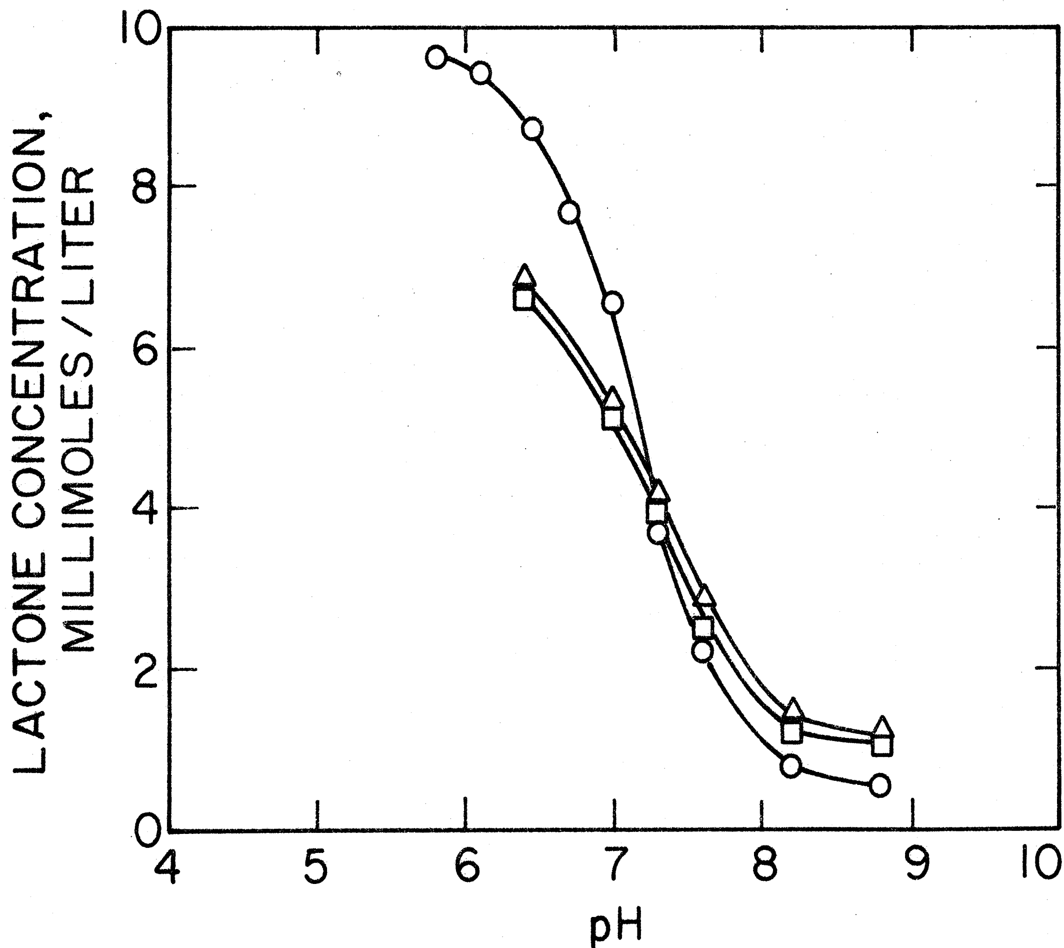


Figure 12. The effect of pH on the rate of gluconic acid production and the lactone concentration in the gluconic acid fermentation by resting cells of Pseudomonas ovalis in 2 liters of Medium B at 25 °C, 315 RPM, 1.0 VVM, and Klett reading 100. Key: ○, Run I, 16 hour culture; □, Run II, and △, Run III, 20 hour cultures.

TABLE VIII

THE EFFECT OF pH ON THE RATE OF GLUCONIC ACID PRODUCTION AND THE STEADY STATE GLUCONOLACTONE CONCENTRATION IN THE GLUCONIC ACID FERMENTATION BY RESTING CELLS OF *Pseudomonas ovalis* IN MEDIUM B. LIQUID VOLUME, 2 LITERS; AERATION RATE, 1.0 VVM; AGITATION RATE, 315 RPM; TEMPERATURE, 25 °C; CELL CONCENTRATION, 100 KLETT UNITS.

Run Number	pH	Gluconic Acid Production Rate, meq/l/hr	Lactone Concentration, mmol/l
I, 16 Hour Culture	5.8	1.47	9.60
	6.1	1.91	9.40
	6.4	2.49	8.70
	6.7	3.08	7.65
	7.0	4.40	6.55
	7.3	4.70	3.65
	7.6	4.11	2.20
	8.2	2.42	0.76
	8.8	1.10	0.50
II, 20 Hour Culture	6.4	2.06	6.60
	7.0	3.60	5.10
	7.3	3.96	3.90
	7.6	4.04	2.50
	8.2	3.82	1.20
	8.8	3.08	1.05
III, 20 Hour Culture	6.4	1.94	6.85
	7.0	3.28	5.26
	7.3	3.67	4.12
	7.6	3.74	2.85
	8.2	3.67	1.43
	8.8	2.89	1.12

concentrations at high and low pH was greater for the 16 hour culture than for the 20 hour cultures. The acid production rates are thus double valued with respect to the lactone concentration, except at the maximum rate.

d) Response of the System to a Step Change in pH.

All fermentations in this report were carried out at pH 7.0, except those used to determine the effect of pH on the rate of acid production. A run was also made to determine the transient response of the system to a step change in pH near the operating pH for most of this study, pH 7.0. For this purpose, a fermentation was run at pH 7.0 until steady state was achieved, then the pH was abruptly changed to 6.7 by adding 10 N H_2SO_4 to the broth. After a new steady state was established at pH 6.7, the pH was quickly changed back to pH 7.0 with 4 N NaOH.

The results are presented in Figure (13) and in Table IX. When the pH was changed to 6.7 there was an immediate drop in acid production rate, followed by a slow recovery to a new steady value, lower than the value previously established at pH 7.0. The lactone concentration rose slowly, the change being linear with respect to time, i.e., an apparent zero-order response, to a new steady value. The time required for the two variables, the acid production rate and the lactone concentration, to reach new steady values was about 50 minutes for each. This was expected, since the acid production rate should be determined by the lactone concentration. Although the response curves took the same amount of time, they did not follow similar paths.

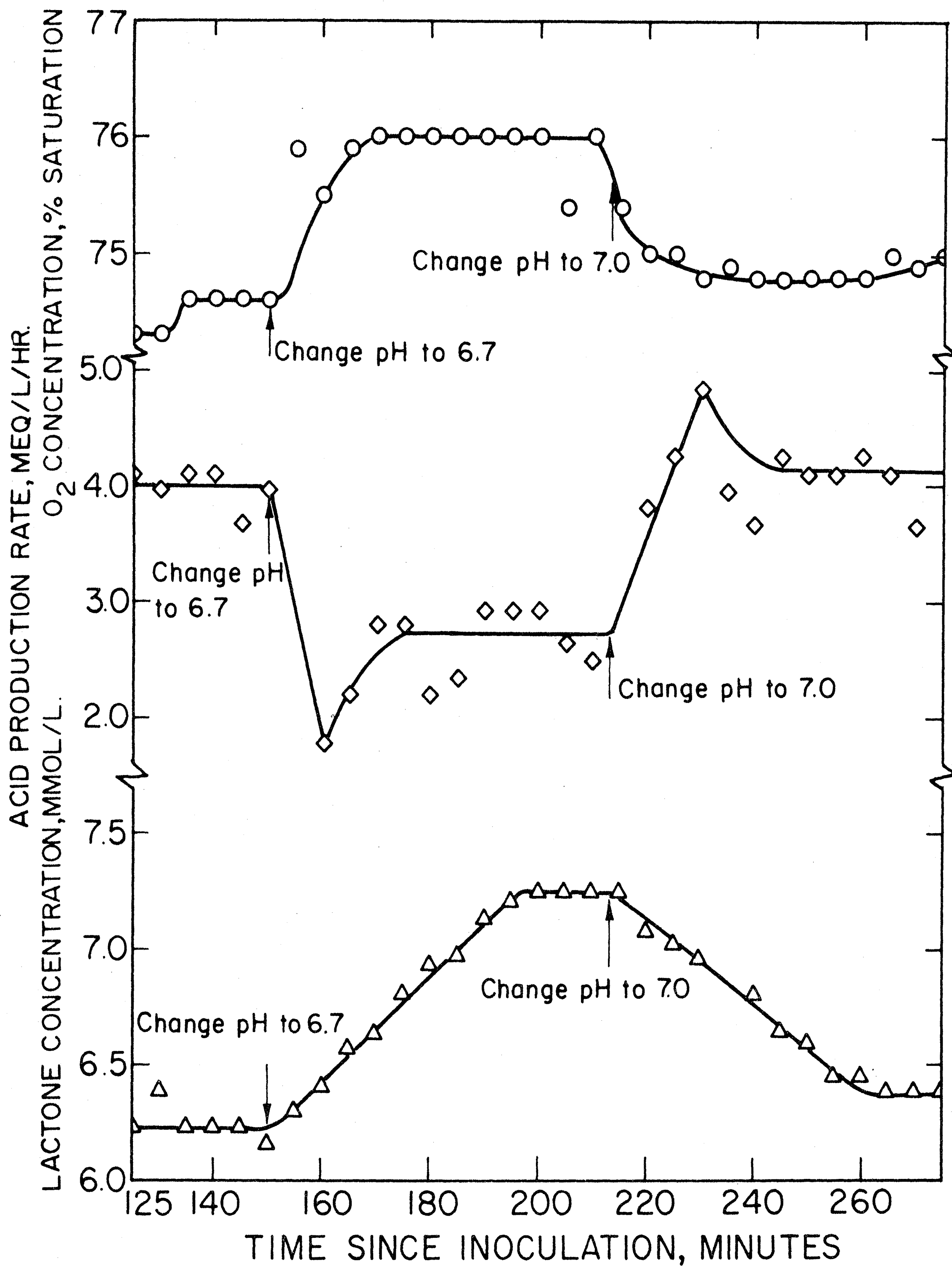


Figure 13. Response of resting cells of Pseudomonas ovalis in the gluconic acid fermentation to a step change in pH. Liquid volume, 2 liters; aeration rate, 1.0 VVM; agitation rate, 315 RPM; temperature, 25 °C; cell concentration, 100 Klett units.

TABLE IX

RESPONSE OF RESTING CELLS OF <u>Pseudomonas</u> <u>ovalis</u> IN THE GLUCONIC ACID FERMENTATION TO A STEP CHANGE IN pH. AERATION RATE, 1.0 VVM; AGITATION RATE, 315 RPM; TEMPERATURE, 25 °C; CELL CONCENTRATION, 100 KLETT UNITS.

Time since Inoculation, min^{-1}	pH	Gluconic Acid Production Rate, meq/l/hr	Lactone Concentration mmol/l	Dissolved O_2 Concentration % Saturation
125	7.0	4.11	6.23	74.3
130		3.96	6.38	74.3
135		4.11	6.23	74.6
140		4.11	6.23	74.6
145		3.67	6.23	74.6
150		3.96	6.16	74.6
155	6.7	--	6.30	75.9
160		1.76	6.40	75.5
165		2.20	6.57	75.9
170		2.79	6.63	76.0
175		2.79	6.81	76.0
180		2.20	6.93	76.0
185		2.35	6.97	76.0
190		2.93	7.13	76.0
195		2.93	7.20	76.0
200		2.93	7.25	76.0
205		2.64	7.25	75.4
210		2.49	7.25	76.0
215	7.0	--	7.25	75.4
220		3.82	7.08	75.0
225		4.26	7.02	75.0
230		4.85	6.96	74.8
235		3.96	--	74.9
240		3.67	6.81	74.8
245		4.26	6.64	74.8
250		4.11	6.60	74.8
255		4.11	6.45	74.8
260		4.26	6.45	74.8
265		4.11	6.38	75.0
270		3.67	6.38	74.9
275		3.96	6.38	75.0

The change in the concentration driving force for absorption, as reflected in the change of the dissolved oxygen concentration, occurred much more rapidly, approaching a new steady value in about 20 minutes. This may be taken as an indication that the response of the bacterial enzyme system was much more rapid than the response of the system as a whole, including presumably purely physical phenomena, such as hydrolysis of the lactone. Note that the percentage drop in the driving force for absorption was considerably smaller than the percentage drop in the acid production rate. Theoretically, they should have been equal. No direct comparison can be made between the change in the acid production rate and the change in the lactone concentration. This is true because the chemical kinetic coefficients change rather rapidly with pH, unlike the mass transfer coefficient, which is presumably not seriously affected by this small change in pH.

When the pH was re-established at 7.0, the effects described above were essentially reversed. There was a rise in the acid production rate, which recovered nearly to its previous value before the pH was lowered to 6.7. A drop in the lactone concentration also occurred, reverting nearly to its value before the pH was changed to 6.7. Both of these changes took place in about 50 minutes. This was approximately the same time as was required for the reverse changes when the pH was lowered to 6.7. The dissolved oxygen concentration dropped to its previous value in about 20 minutes, again indicating that changes in oxygen uptake occur much more rapidly than changes in the acid production rate.

e) The Effect of Gas-Liquid Interfacial Area on the Hydrolysis of the Intermediate Gluconolactone.

Bennett and Kempe (11) suggested that elimination of one of the absorption resistances might account for their finding that oxygen uptake rates were larger in the presence of bubbles than in their absence. As pointed out in the Introduction to this thesis, this not a promising explanation because resistance to absorption through the liquid film around the cells is negligibly small. In any case, it was shown in the experiments described above that the difference was an artifact. The two rates are equal at steady state. Nevertheless, an increase in the bulk lactone concentration, and an increase in the acid production rate, were found to accompany an increase in the agitation rate. It is important to note, however, that the rate of oxygen depletion occurring after aeration was stopped was still independent of the dissolved oxygen concentration. Also, the oxygen depletion rate increased as the lactone concentration increased following an increase in the agitation rate. No current theories can explain this phenomenon. In fact, it was shown in the Introduction to this thesis that, according to current concepts, increased agitation should have no effect whatsoever on oxygen uptake rates.

One of the consequences of increased agitation is, as Bennett and Kempe pointed out, an increased gas-liquid interfacial area. A series of experiments was designed to see whether the interfacial area has any influence on the chemistry of the fermentation, apart from any interfacial effects on oxygen absorption. In these experiments, a pseudo-steady state was achieved in a fermentation in the

usual fashion. Then the air supply was cut off. After the dissolved oxygen tension had fallen to a low value, the lactone concentration was measured as a function of time for two cases: 1) with nitrogen bubbled through the broth at 1.0 VVM, the same rate as air had previously been bubbled through, and 2) with nitrogen passed through the head space of the fermentor. In either case all molecular oxygen was removed from the system so there was no oxygen absorption taking place, and mass transfer was eliminated. Rates of acid production as measured by the rate of alkali addition at constant pH were also measured. The experiments were repeated at various agitation rates.

It was found in all cases that the rate of disappearance of the lactone could be represented by an equation of the type

$$-d\left[L\right]/dt = k\left[L\right]$$

where $\left[L\right]$ is the concentration of lactone, and k is a constant. Typical curves, both with and without nitrogen bubbles, are shown in Figure (14). The equation is of the same form as that derived in the Discussion from a kinetic model used to describe the system for these conditions. Results of the experiments are given in Table X, where $k \equiv K_9$ for the case where nitrogen was passed through the head space, and $k \equiv K_9'$ for the case where nitrogen was bubbled through the broth. It was consistently found that the lactone disappeared faster when nitrogen bubbles were present than when nitrogen was merely passed through the head space. Thus it appears that the presence of a large interfacial area has a direct effect on the kinetics of the chemical or biochemical reactions of the system. The data are

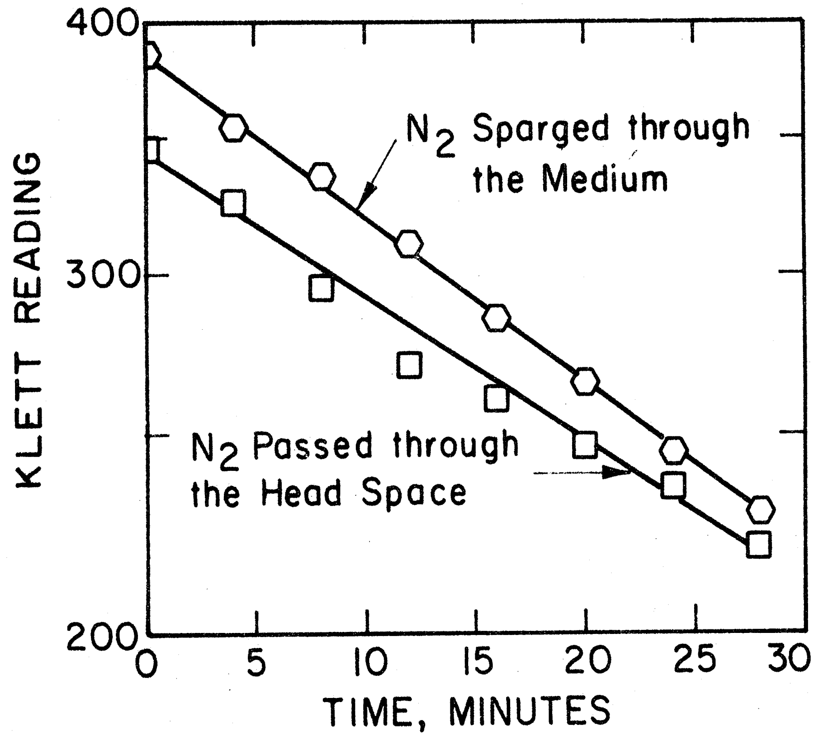


Figure 14. Rate of disappearance of gluconolactone from a fermentation broth in the gluconic acid fermentation of Pseudomonas ovalis after the air supply had been cut off. Liquid volume, 2 liters; agitation rate, 310 RPM; temperature, 25 °C; cell concentration, 100 Klett units.

TABLE X

COEFFICIENTS FOR THE DISAPPEARANCE OF LACTONE FROM MEDIUM B IN THE ABSENCE OF MOLECULAR OXYGEN FOR THE GLUCONIC ACID FERMENTATION BY RESTING CELLS OF *Pseudomonas ovalis*. LIQUID VOLUME, 2 LITERS; pH, 7.0; TEMPERATURE, 25 °C; CELL CONCENTRATION, 100 KLETT UNITS. THE COEFFICIENTS ARE DEFINED IN THE TEXT.

Agitation Rate, RPM	$K_9 \times 10^2$ Nitrogen in Head Space	$K_9' \times 10^2$ Nitrogen Bubbles	$k_5 \times 10^2$ Nitrogen in Head Space	$k_5' \times 10^2$ Nitrogen Bubbles	$k_5^{O2} \times 10^2$ From the Steady State
205	1.73	1.74	1.14	1.12	1.25
253	1.61	1.78	1.01	0.92	1.16
255	1.67	1.79	1.03	1.01	1.42
273	1.60	1.75	0.92	0.95	1.38
273	1.83	2.00	1.11	1.02	1.30
273	1.87	1.93	1.19	0.98	1.27
310	1.60	1.87	1.11	1.01	1.53
311	1.53	1.67	0.97	0.81	1.23
345	1.59	1.86	1.09	0.90	1.27
345	—	1.83	—	1.05	1.41

imprecise, and no good correlation is possible. Nevertheless, there is a general tendency for the difference between K_9 and K_9' to become larger at higher agitation speeds.

The rate of acid formation could also be expressed by an equation of the type

$$d\left[A\right]/dt = k\left[L\right]$$

where $\left[A\right]$ is the concentration of gluconate ion. For acid production, the kinetic constants are represented as k_5 and k_5' for the case of no bubbles and the case with bubbles present, respectively, in accordance with the model developed in the Discussion. In general, the rate of acid production was not the same as the rate of lactone disappearance, so $K_9 \neq k_5$, and $K_9' \neq k_5'$. The kinetic constants for acid formation are smaller than the corresponding constants for lactone disappearance, indicating that not all of the lactone originally present in the broth when the air supply was cut off was hydrolyzed to gluconic acid. Moreover, whereas overall lactone disappearance was faster in the presence of bubbles, the opposite was true for acid production. The kinetic constant k_5' for the acid formation rate as a function of lactone concentration in the presence of nitrogen bubbles was found to be smaller than k_5 for the acid formation rate in the absence of bubbles, with but a single exception.

Finally, $k_5^{O_2}$, the kinetic coefficient for acid formation as a function of lactone concentration during the steady state with air bubbles present was larger in every case than k_5 or k_5', the coefficients calculated from the transient experiments with no molecular oxygen present.

IV. DISCUSSION

Aerobic fermentations are usually found to proceed at a rate that is independent of dissolved oxygen activity in the fermentation medium, provided the activity is above a "critical" value which is characteristic of the organism involved. There are a number of ways of interpreting this phenomenon. Winzler (86), in a series of elegant experiments with yeast cells, hypothesized that the effect could be attributed to saturation of the enzyme surface. This is tantamount to saying that chemical reaction potentials are limiting rather than oxygen transfer potentials. Others have attributed the phenomenon of the "critical oxygen concentration" to oxygen transfer effects, or to fluid mixing effects (51, 76).

In the Introduction to this thesis, the conditions were outlined under which a gas-liquid absorber must be limited by chemical reaction rates. An absorber with zero order chemical reaction with respect to the absorbed gas will be limited by chemical reaction when the concentration of the absorbed gas in the liquid lies between the physical equilibrium concentration in the absence of chemical reaction and the concentration at chemical equilibrium. This theoretical concept is in agreement with the empirically based hypothesis of Winzler. Such absorbers are described according to the classification scheme of Astarita (7) as operating in the kinetic subregime of the slow reaction regime. The theoretical concept is correct for an aerobic fermentation whenever the reaction is zero order or pseudo-zero order with respect to the dissolved oxygen concentration. Before such a

hypothesis can be accepted for a particular fermentation, it must be shown that this truly obtains, and that some other factor is not responsible for the reaction appearing to be zero order. Aside from the possibility of chemical inhibition, the most likely factors would be poor fluid mixing, a large resistance to diffusion of one of the reactants from the bulk fluid to the enzyme loci, or a large resistance to diffusion of products away from the enzyme loci. Poor mixing is rather easy to avoid in the usual laboratory scale fermentors, although mixing could conceivably become limiting in very large industrial fermentors. Finn (31) was probably correct for most cases in his claim that mixing sufficient to keep the cells individually suspended will be adequate. It has been shown in the Introduction to this thesis that the diffusion transfer coefficients to and from the cells are usually several orders of magnitude larger than the coefficients for absorption from the gas phase to the bulk liquid. On this basis, it is probable that most fermentations which appear to operate with a critical concentration actually do have a critical concentration. They may be said to operate in the kinetic subregime of Astarita.

However, a number of cases from the literature were cited in the Introduction to this thesis for which increases in fermentation rates were obtained by various means, even though the dissolved oxygen concentrations were above the critical concentrations generally accepted. No satisfactory explanation has yet appeared for any of these reported effects. One noteworthy instance of a fermentation in which such an effect has been observed is the gluconic acid

fermentation of Pseudomonas ovalis. Tsao and Kempe (79) first observed an increase in the rate of gluconic acid production by resting cells of this organism with an increase in the agitation rate of their fermentor. They did not report dissolved oxygen levels, and it was not known whether the dissolved oxygen content was above the critical.

Aiba, Hara, and Someya (2) measured oxygen uptake by resting cells of P. ovalis in a bubble aeration column without agitation. They measured uptake rates by two different methods. They found that the oxygen uptake rate as measured by gluconic acid production in the presence of air bubbles was higher than the rate of oxygen uptake of a sample of their broth measured polarographically. The oxygen concentration was above the critical level for all of their experiments.

Bennett and Kempe (11) set out to determine whether the effects described by Tsao and Kempe could still be detected when the dissolved oxygen concentration was known to be above the critical. They found once again that an increase in the gluconic acid production rate resulted from an increase in the agitation rate, even though the critical dissolved oxygen concentration was exceeded at all times. In addition, at each agitation speed Bennett and Kempe measured the rate of oxygen depletion in their fermentation broth after the air supply had been cut off. The rates were measured by means of a membrane type electrode. They found that the oxygen depletion rate was independent of the stirring rate and was lower than the corresponding steady state acid production rates established at each agitation rate.

The accumulation of significant amounts of an intermediate gluconolactone was detected for this fermentation at pH 5.8 by Humphrey

and Reilly (41). They also determined that *P. ovalis* does not produce a lactonase, so hydrolysis of the lactone to gluconic acid is spontaneous. The experiments of Tsao and Kempe and of Bennett and Kempe were carried out at pH 7.0. The nonenzymatic hydrolysis of the lactone is much more rapid at pH 7.0 than at pH 5.8. Nevertheless, if lactone had accumulated in the broth during the steady state acid production experiments of Bennett and Kempe, then they were not necessarily measuring the instantaneous rates of oxygen uptake, but rather the rate of spontaneous hydrolysis of the lactone. Only if the lactone concentration were constant would the oxygen uptake and the gluconic acid production rates be the same.

In experiments reported in this thesis it was shown that a pseudo-steady state could be achieved in which the dissolved oxygen level, the lactone concentration, and the acid production rate were all constant, provided the temperature, pH, aeration rate, and agitation rate were also constant. When the agitation rate was increased the acid production rate rose, as reported by Bennett and Kempe. In addition the gluconolactone concentration rose. Thus, on a simple phenomenological level, the reason the acid production rate increased when the agitation rate was increased was because the lactone level increased, and the rate of acid production is directly proportional to the lactone concentration. On a theoretical level, of course, this finding merely shifts the emphasis, since the problem changes from determining the reason for the increase in acid production rate to that of explaining the increase in the lactone concentration.

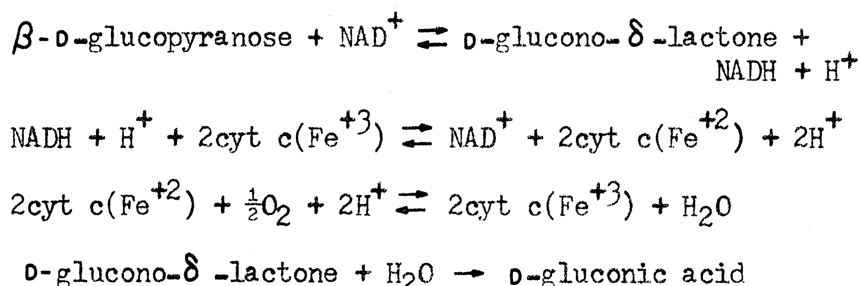
In the transient experiments it was found that the rate of oxygen depletion in the broth after the air supply was cut off was independent of the dissolved oxygen level down to roughly 25 per cent of saturation. On the other hand, the rate was dependent on the lactone concentration in the broth. That is, the slopes of plots of dissolved oxygen as a function of time after the air supply was cut off increased with increased agitation rate. Thus, from one point of view, it can be stated that a critical oxygen concentration does exist, but the _caveat_ must be added that it is observable only at a constant, or very nearly constant, lactone concentration.

Phenomenologically, then, the gluconic acid fermentation of _P. ovalis_ can be characterized by the following:

1. Ultimately, the fermentation rate is determined by the net rate of lactone formation.

2. Above a certain value, which was about 25 per cent of saturation for the case at hand, the rate of oxygen uptake is independent of the dissolved oxygen concentration.

In the following, a kinetic model will be derived which predicts this sort of behavior. A reaction scheme was proposed in the Introduction to this thesis based on information from the literature for glucose dehydrogenase systems from other organisms. The scheme is presented again here for convenience.



$$\beta\text{-D-glucopyranose} + NAD^{+} \rightleftharpoons \text{D-glucono-}\delta\text{-lactone} + NADH + H^{+}$$

$$NADH + H^{+} + 2\text{cyt } c(Fe^{+3}) \rightleftharpoons NAD^{+} + 2\text{cyt } c(Fe^{+2}) + 2H^{+}$$

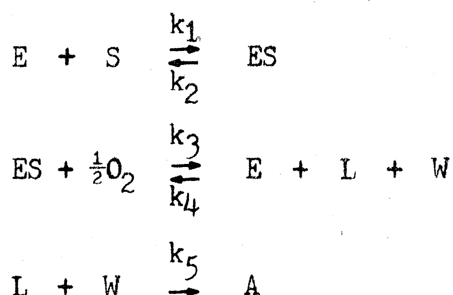
$$2\text{cyt } c(Fe^{+2}) + \tfrac{1}{2}O_2 + 2H^{+} \rightleftharpoons 2\text{cyt } c(Fe^{+3}) + H_2O$$

$$\text{D-glucono-}\delta\text{-lactone} + H_2O \rightarrow \text{D-gluconic acid}$$

Overall:

$$\text{D-glucose} + \tfrac{1}{2}O_2 \rightarrow \text{D-gluconic acid}$$

Each of these reactions, except the hydrolysis of the lactone, is presumably mediated by a specific enzyme. Therefore, a kinetic model specifically incorporating all of these reactions would be most unwieldy, if not impossible, with present techniques. The cytochrome functions as an electron carrier, and does the NAD^+. If it is assumed that formation of an enzyme-sugar complex is slower than the subsequent transfers of electrons or protons, then the following highly simplified reaction scheme can be proposed for the conversion of glucose to gluconic acid by P. ovalis.



$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$$

$$ES + \tfrac{1}{2}O_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + L + W$$

$$L + W \xrightarrow{k_5} A$$

E is the free enzyme, S is sugar, ES is an activated enzyme-sugar complex, O_2 is oxygen, L is the gluconolactone, W is water, and A is the product, gluconic acid. The reaction model ignores all transfers of electrons and protons, which amounts to assuming that such transfers are infinitely fast. The first two reactions are assumed reversible. For this assumption to be justified, it is necessary that all of the reaction steps in the actual sequence of reactions be reversible, with the exception of the hydrolysis of the lactone to gluconic acid. The latter is nonenzymatic and almost completely irreversible at

physiological pH values. The reversibility of cytochrome c has been amply demonstrated as was already mentioned (29, 36, 52). Strecker and Korkes (73) demonstrated that the lactone formation reaction is easily reversible. The simplified reaction scheme given here is therefore feasible at least with respect to the reversibility of the reactions.

In writing rate equations for enzyme systems, it is a standard practice to assume that the free enzyme concentration and the concentration of the enzyme-substrate complex rapidly approach constant values. Thus the net rate of formation of the activated complex can be written as

$$\frac{d[ES]}{dt} = 0 = k_1[E][S] - (k_2 + k_3[O_2]^{\frac{1}{2}})[ES] + k_4[E][L][W] \tag{30}$$

Since there is no growth of cells in the medium it can be assumed that the total enzyme concentration is a constant:

$$[E]_o = [E] + [ES] \tag{31}$$

Equations (30) and (31) can be rearranged and combined to give $[E]$ and $[ES]$ in terms of $[E]_0$:

$$[E] = \frac{k_2 + k_3[O_2]^{\frac{1}{2}}}{k_1[S] + k_2 + k_3[O_2]^{\frac{1}{2}} + k_4[L][W]} [E]_o \tag{32}$$

$$[ES] = \frac{k_1[S] + k_4[L][W]}{k_1[S] + k_2 + k_3[O_2]^{\frac{1}{2}} + k_4[L][W]} [E]_o \tag{33}$$

Under pseudo-steady state conditions $d[L]/dt$ is zero, so that, if it is assumed that $[L]^2 << [L]$, and that $[W]$ can be replaced with activity units, with $a_W = 1$, an expression for the lactone concentration

can be written, making use of Equations (32) and (33):

$$[L] = \frac{k_1 k_3 [E]_o}{\frac{k_2 k_4 [E]_o}{[S][O_2]^{\frac{1}{2}}} \quad \frac{k_1 k_5}{[O_2]^{\frac{1}{2}}} \quad \frac{k_2 k_5}{[S][O_2]^{\frac{1}{2}}} \quad \frac{k_3 k_5}{[S]}} \qquad (34)$$

If it is assumed that the sugar is present in large excess, and hence is of essentially constant concentration, then

$$[L] = \frac{K_3}{\frac{K_1}{[O_2]^{\frac{1}{2}}} + K_2} = \frac{K_3 [O_2]^{\frac{1}{2}}}{K_1 + K_2 [O_2]^{\frac{1}{2}}} \qquad (35)$$

The rate of product formation can thus be written

$$\frac{d[A]}{dt} = k_5[L][W] = k_5[L] = \frac{K_4 [O_2]^{\frac{1}{2}}}{K_1 + K_2 [O_2]^{\frac{1}{2}}} \qquad (36)$$

If $K_2(O_2)^{\frac{1}{2}} >> K_1$ then

$$\frac{d[A]}{dt} = \frac{K_4}{K_2} = K_5 = k_1[E]_o[S] = k_5[L] \qquad (37)$$

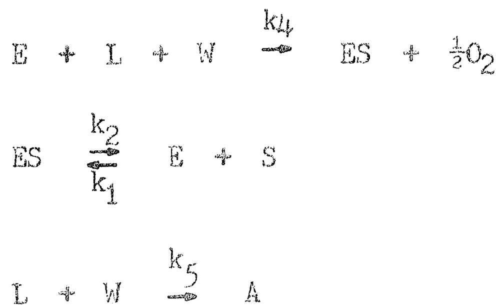
since $K_4 = k_5 K_3 = k_1 k_3 k_5 [E]_o$ and $K_2 = k_3 k_5/[S]$.

Equations (36) and (37) may be compared with the phonomenological characteristics of the gluconic acid fermentation pointed out above. First, Equation (36) predicts that provided K_2 is sufficiently large, the rate of acid production will be independent of the dissolved oxygen concentration above some value determined by the kinetic constants of the enzyme system. This is in agreement with the experimental results. This concentration may be identified with the classical "critical" oxygen concentration. Its formation agrees with the

hypothesis of Winzler (86) that the critical concentration is determined by the chemistry of the enzyme system rather than by physical factors.

Second, Equation (37) predicts that the controlling reaction when the dissolved oxygen concentration is above the critical will be the rate of formation of the activated enzyme-glucose complex.

The model given here can also be used to predict the form of the equation describing the rate of disappearance of lactone from the broth when the air supply is cut off. Assume the case wherein a pseudo-steady state is achieved, and then the air supply is suddenly cut off, and nitrogen gas is passed through or over the fermentation medium. After all the oxygen has been consumed metabolically or stripped away the following reactions remain from the scheme given above:



$$E + L + W \xrightarrow{k_4} ES + \tfrac{1}{2}O_2$$

$$ES \underset{k_1}{\overset{k_2}{\rightleftharpoons}} E + S$$

$$L + W \xrightarrow{k_5} A$$

The reaction $ES + \frac{1}{2}O_2 \rightarrow E + L + W$ need not be considered if the assumption is made that oxygen molecules are stripped away by the nitrogen as soon as they are produced. Note that the overall effect of this scheme is a competition between the non-enzymatic hydrolysis of the lactone to gluconic acid and the regeneration of glucose by reversed electron flow through the cytochrome c and nucleotide carriers.

The rate of disappearance of lactone can be written, assuming $a_W = 1$:

$$-\frac{d[L]}{dt} = k_4[E][L] + k_5[L] \tag{38}$$

Proceeding as before, $[E]$ can be expressed in terms of $[E]_o$

$$[E] = \frac{k_2[E]_o}{k_1[S] + k_2 + k_4[L]} \tag{39}$$

Substituting Equation (39) in Equation (38)

$$-\frac{d[L]}{dt} = \frac{k_2 k_4 [E]_o [L]}{k_1[S] + k_2 + k_4[L]} + k_5[L] \tag{40}$$

Once again, assuming constant $[S]$

$$-\frac{d[L]}{dt} = \frac{K_6[L]}{K_7 + k_4[L]} + k_5[L] \tag{41}$$

If $k_4[L] >> K_7$

$$-\frac{d[L]}{dt} = \frac{K_6}{k_4} + k_5[L] = K_8 + k_5[L] = k_2[E]_o + k_5[L] \tag{42}$$

If $K_7 >> k_4[L]$

$$-\frac{d[L]}{dt} = \frac{K_6}{K_7}[L] + k_5[L] = K_9[L] \tag{43}$$

Finally, the rate of acid production for the conditions under consideration is simply

$$\frac{d[A]}{dt} = k_5[L] \tag{44}$$

In Table II of the Experimental Results section values of the kinetic constants K_9 and k_5 are given. Data for these calculations

were collected by cutting off the air supply to a fermentation and measuring the rate of disappearance of lactone from the broth. Primed constants designate the cases where nitrogen was bubbled through the broth, and unprimed constants designate the cases where nitrogen was passed through the head space of the fermentor.

The model developed in this Discussion appears to be satisfactory for describing the effects of oxygen concentration and lactone concentration on the rate of gluconic acid formation by resting cells of *P. ovalis*. These effects are that at a given steady lactone concentration, the rate of oxygen uptake, and hence the rate of acid production, will be independent of the dissolved oxygen concentration above a certain level. Manipulation of the interphase oxygen transfer driving force will have no effect on the fermentation if all other factors remain the same. This was shown to be true by Bennett (10), who varied the partial pressure of oxygen in the gas fed to his fermentor by bleeding nitrogen into the feed air. The total gas flow rate was constant. In this way the dissolved oxygen level could be varied at constant agitation rate and gas flow rate. Inspection of Bennett's data for these experiments shows that above a characteristic dissolved oxygen level the rate of acid production became independent of dissolved oxygen.

All of the above is consistent with the widely accepted concept of the "critical" oxygen concentration. However, with this fermentation it was also found that acid production rates could be increased by increasing the agitation rate, even when the dissolved oxygen concentration was always above the critical. The phenomenon has been

previously reported by Bennett and Kempe (11). They also found that although steady state acid production rates increased when the agitation rate was increased, the rate of oxygen uptake as measured by oxygen depletion rates after the air supply had been cut off was independent of both the agitation rate and the dissolved oxygen concentration, when that concentration was above the critical. Subsequent experiments reported herein have shown the latter to be an artifact. The transient rates as well as steady state rates increased with increasing agitation intensity. It is probable that Bennett and Kempe, unaware of the accumulation of the intermediate gluconolactone, did not allow sufficient time to elapse between their transient measurements for the lactone concentration to change significantly. In experiments reported in this thesis, the minimum time required to establish a new steady state after changing the agitation rate was one hour or more. Therefore, Bennett and Kempe did not observe any changes in the oxygen depletion rate.

Nevertheless, the fact remains that increasing the agitation rate increased the fermentation rate in the experiments reported in both Bennett's thesis and in this thesis, although the oxygen concentration was always above the critical. This was found to be true whether the fermentation rate was measured by the steady state acid production rate, or by the transient oxygen depletion rates after the gas supply was cut off. Thus, the rates measured by dissolved oxygen depletion increased with increasing agitation, provided a new steady state acid production rate, and hence a new lactone concentration, was established prior to each transient measurement. At the same

time, all of the transient measurements were independent of the dissolved oxygen concentration. That is, plots of dissolved oxygen as a function of time after the air supply was cut off were linear for each agitation rate. A correction for reaeration through the surface was applied to these data. This result is in agreement with Equation (36). The slopes of the plots, however, increased with increased agitation rate.

Theoretically, if the rate of oxygen uptake is independent of the dissolved oxygen concentration then, for an absorber of this type, increasing the agitation rate should have no effect on the absorption rate or the fermentation rate. Bennett and Kempe suggested that the reason this did not obtain for the gluconic acid fermentation was due to the existence of two parallel paths for transfer of oxygen from air to the cells. One path is through the resistances listed in the Introduction, as suggested by Bartholomew _et al_. (8). The other path suggested by Bennett and Kempe was also first pointed out by Bartholomew and his co-workers. In this path, some of the cells are assumed to adsorb on the surfaces of the air bubbles as they pass through the liquid. In this way, one of the liquid film resistances and the bulk liquid resistance are eliminated for a portion of the cells. It has been shown, however, that the resistances of the bulk liquid and the liquid-cell film are negligible compared to the resistance of the liquid film around the bubble (31, 55). Therefore, elimination of the former would have no effect on the fermentation, even if mass transfer were controlling. In any case, it is evident from Astarita's work (7) that chemical reaction is the controlling

process for a fermentation of this type, provided that the dissolved oxygen level is maintained above the critical.

The dependence of the fermentation rate on the agitation rate, even though oxygen transfer is not limiting, seems to indicate that the surfaces of the bubbles, that is the gas-liquid interface, may be an important factor. If this is so, then since the fermentation is controlled by chemical or biochemical reaction rates, the effect would have to be due to an increase in the rate of at least one of the chemical reactions in the system. This change would have to result from the presence of the interface. Experiments have been described in which the rate of disappearance of the chemical intermediate gluconolactone was measured in the absence of any oxygen transfer. This rate was larger when bubbles of the inert gas nitrogen were passed through the broth than it was when nitrogen was passed only over the top of the broth. No oxygen was present in either case, so oxygen transfer was eliminated as a factor in these experiments. Thus the surfaces of the bubbles seem to afford an increased reaction rate over that observed in the bulk solution.

Before proceeding to an analysis of this observation, a brief discussion of the physico-chemical factors which might allow an increase in a chemical reaction at a gas-liquid interface will first be made.

The energies of activation for reactions in the bulk phase and in surface layers are usually equal. The only known exceptions are found in films which expand at a constant surface pressure over a limited temperature range. The expansion of the film will provide an

increased accessibility of reactive groups in the film molecules (22).

A second possibility is steric factors. However, such effects are confined to dense monolayers for the most part. In addition, they almost always lead to reductions in reaction rates over those in bulk fluids (23).

An interesting way in which it is apparently possible to alter chemical reactions at a gas-liquid interface was suggested recently by Sprow and Prausnitz (70). They pointed out that substances which are surface active will tend to concentrate at the gas-liquid interface. The activities of such substances will in general be different from the activities in the bulk solution. The difference in activity of a surface active compound in a surface film from its activity in the bulk solution can be expressed by (28)

$$\gamma = \frac{A_i}{\bar{A}_i} \gamma_i + \frac{RT}{\bar{A}_i} \ln \frac{a_i^{\sigma}}{a_i^{b}}$$

Here γ represents the surface tension of the mixture, γ_i the surface tension of pure component i, A_i the molar surface area of component i, and $\bar{A}_i$ the partial molar surface of component i in the surface solution.

Sprow and Prausnitz pointed out that if the surface active components in a solution are capable of undergoing a chemical reaction, the equilibrium constant for that reaction in the surface will in general be different from the equilibrium constant in the bulk solution. This is because the activities in the surface phase are different. Butler (14) derived an equation for the ratio of the equilibrium constant in the surface phase to the equilibrium constant

in the bulk phase. The concept has been reviewed more recently by Eriksson (28). The ratio of constants is given by

$$\ln(K^{\sigma}/K^{b}) = \frac{1}{RT}\sum_i \nu_i \left(\bar{A}_i \gamma - A_i \gamma_i\right)$$

where the ν_i are the stoichiometric coefficients

$$\sum_i \nu_i B_i = 0$$

The B_i are the reacting species.

The shift in equilibrium will be favorable if the products of the reaction have a lower surface tension than the reactants. For example, Sprow and Prausnitz (70) gave sample calculations for the hydrolysis of ethyl acetate at 20 °C. They calculated that in a dilute solution such that $\gamma = 65$ dynes/cm, the equilibrium constant in the surface phase would be about 140 times the equilibrium constant in the bulk solution. This is a large increase, and even a relatively small ratio of surface phase volume to bulk volume could bring about a significant improvement in the equilibrium yield.

Sprow and Prausnitz suggested that better yields could be obtained for certain reactions by dispersing the liquid to produce a high surface-to-volume ratio. While this is theoretically correct, it would still be necessary to return to a lower surface-to-volume ratio eventually, in order to extract the products. When this was done, the usual equilibrium constant obtained in the bulk would prevail, and the reaction would proceed in the reverse direction. This problem could be overcome only if the reaction rates were sufficiently slow to allow the recovery of products before the reverse reaction had proceeded to an appreciable extent.

Superficially, it would seem that if the equilibrium constant is altered in the surface phase then the kinetic coefficients for the reaction would be changed, also. This would seem to be so since the equilibrium constant for a chemical reaction can be expressed as a ratio of the chemical kinetic coefficients. However, it has been pointed out that the kinetic rate constants are altered in the surface phase only for certain very restricted cases (22).

The equilibrium in the surface phase is not simply a chemical equilibrium. There is also an exchange equilibrium for surface active chemical species between the surface phase and the bulk phase. If the products of a chemical reaction are highly surface active, then they will be adsorbed into the surface phase. The products in the surface phase will then be in excess, causing the formation of the original reactants. The original reactants, if they are not strongly adsorbed, will desorb into the bulk phase. This will upset the equilibrium in the bulk phase, causing the formation of more of the products. This will cause more of the products to adsorb into the surface phase, and so on. This cyclic process will be superimposed on the normal chemical equilibrium. If the rates of the various processes are favorable, then the concentrations of products in the surface film will be considerably higher than would be found at chemical equilibrium in the bulk phase.

It is probably some such process as this that causes the equilibrium concentrations in a surface film to be displaced from the equilibrium concentrations in the bulk phase. The process does not bring about any change in the rate constants for the reaction.

Therefore, a phenomenon of this sort could not be responsible for the increase in reaction rates observed in surface films for the gluconic acid fermentation.

Another effect in the surface layer is one which might possibly result from the net charge on the surface of bacteria. When bacteria are free in the bulk of a solution, the electrostatic lines of force due to the surface charge of the bacteria are more or less symmetrically arranged. When cells are at the interface, the gas phase acts as an electrical insulator, and the lines of force are directed into the liquid. Very large electrical gradients may be set up in this manner, which may attain local values of one million volts per centimeter. The effect is often important relative to thermal energy at distances as great as several hundred Angstroms from the surface (23).

The pertinent effect arising from cells or any other particles bearing a net charge being in the vicinity of the gas-liquid interface is to change the concentration of ions near the interface, most importantly the pH. Quantitatively, the changes are expressed by (23):

$$\left[OH^-\right]^\sigma = \left[OH^-\right]^b \exp(+\epsilon\psi/kT) \qquad (45)$$

$$\left[H^+\right]^\sigma = \left[H^+\right]^b \exp(-\epsilon\psi/kT) \qquad (46)$$

The superscript σ refers to conditions in the surface phase, and the superscript b refers to conditions in the bulk phase. The symbol ϵ represents the electronic charge in e.s.u., and ψ is the potential due to the charged particles at the interface.

Inspection of Equations (45) and (46) reveals that if the particles at the surface bear a net negative charge, then the pH in the

surface phase will be lower than the pH in the bulk phase. Conversely, if the net charge on the particles is positive, then the pH in the surface phase will be higher than the pH in the bulk phase. Such changes in pH can be rather large in some cases. Peters (59) found that half-ionization of a long chain acid adsorbed at the interface between benzene and water occurred about three pH units to the alkaline side of the point at which such materials are half-ionized in aqueous solution. On the other hand, amines are apparently half-ionized in the surface at about 3 or 4 pH units to the acid side of the point of half-ionization in bulk aqueous solution.

For changes in the soluble ion concentration to occur at an interface it is only necessary that charged particles be found at that interface. However, the effect will be enhanced if there is an excess concentration of particles at the surface relative to the bulk liquid. Such an excess of particles could produce charge densities many times greater than would be possible in the bulk solution. An excess concentration of particles in the surface phase will occur if the particles become adsorbed at the gas-liquid interface. Bennett and Kempe (11) hypothesized adsorption of P. ovalis cells on gas bubble surfaces in attempting to explain the increase in the gluconic acid fermentation rate which they observed when the agitation rate was increased. They suggested that since cells of P. ovalis could be concentrated by foam fractionation, then possibly the cells also concentrated in the liquid film around gas bubbles in their fermentor.

Gibbs (33) first showed that adsorption of charged particles at a gas-liquid interface could be related to the interfacial tension

between the phases. He derived an equation from thermodynamic considerations relating surface tension to the concentration of particles in the interface. For the adsorption of bacteria in the presence of excess salts the equation can be written as

$$- C_B \left(\frac{\partial \gamma}{\partial C_B}\right)_T = kTn \qquad (47)$$

Equation (47), which is one form of the so-called Gibbs adsorption isotherm, is derived in Appendix A. Here C_B is the concentration of bacterial cells in the bulk liquid, γ is the interfacial tension, and n is the excess concentration of cells in the surface phase, relative to the bulk liquid cell concentration. It follows from Equation (47) that if the presence of cells in the liquid brings about a depression of the gas-liquid interfacial tension, then n is finite. Therefore, adsorption of cells at the gas-liquid interface takes place.

Depression of the gas-liquid interfacial tension by cells of *P. ovalis* is only indirect evidence of adsorption. Observation of such an effect by no means constitutes proof that adsorption of cells occurs. Nevertheless, a simple experiment was conducted in the following manner. Cells of *P. ovalis* were grown in Medium A on the shaker for 18 hours. Then the cells were centrifuged from the broth, washed once with distilled water, and resuspended in a small amount of Medium B. For this experiment the antifoam was omitted from Medium B. Suspensions of various cell concentrations were made by adding small portions of this suspension to a larger amount of Medium B. The gas-liquid interfacial tension was measured at each

cell concentration with a duNouy ring tensiometer. Figure (15) shows the variation of gas-liquid interfacial tension for such suspensions as a function of bacterial cell concentration. The concentration of bacteria is given in terms of the readings from a Klett-Summerson photocolorimeter using a blue, 420 mμ filter. Such readings are a linear function of concentration up to a reading of about 200 Klett units.

Figure (16) is a plot of $-C_B(\partial\gamma/\partial C_B)_T$ versus the Klett reading for the data of Figure (15), in accordance with Equation (47). It is important to remember that the ordinate is a measure of the excess surface concentration of cells, not the absolute concentration. The drop in the excess surface concentration at the higher Klett readings does not mean that the absolute surface concentration is dropping. Rather it indicates that the surface concentration is approaching a constant value. This has been shown to be true by Nilsson (57) for sodium lauryl sulphate ions.

That adsorption of cells of *P. ovalis* takes place at the gas-liquid interface is at least indirectly indicated from the interfacial tension data given. Cells of *P. ovalis* are negatively charged at pH values above the isoelectric point. The isoelectric point of *P. ovalis* was determined by Daniels (21) to be 3.9. According to Equation (46), the pH will be lowered in the vicinity of any cells which are adsorbed at the bubble surfaces in a sparged gluconic acid fermentation, when the bulk pH is above about 4.0. Assuming that adsorption of cells does occur, resulting in a lowered pH in the liquid

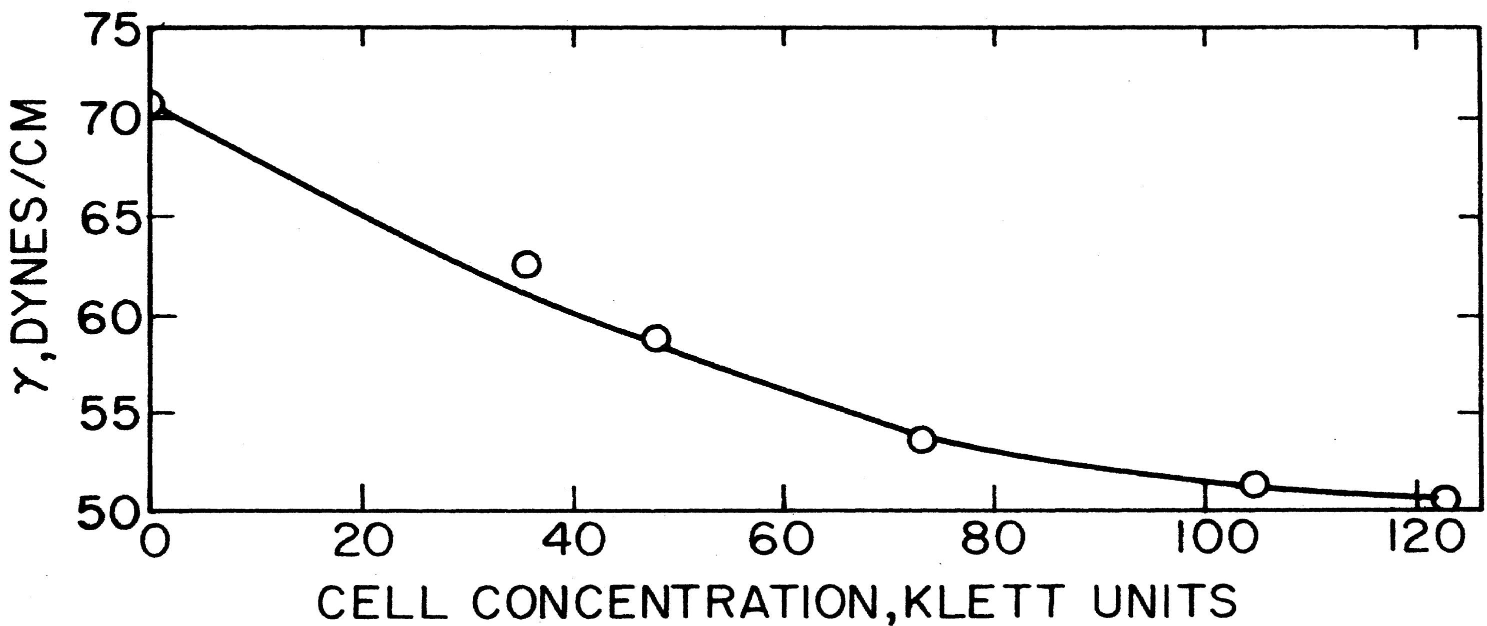


Figure 15. The effect of bacterial concentration on the interfacial tension of suspensions of *Pseudomonas ovalis* in Medium B against air. No antifoam was present. Temperature, 25 °C; pH 7.0.

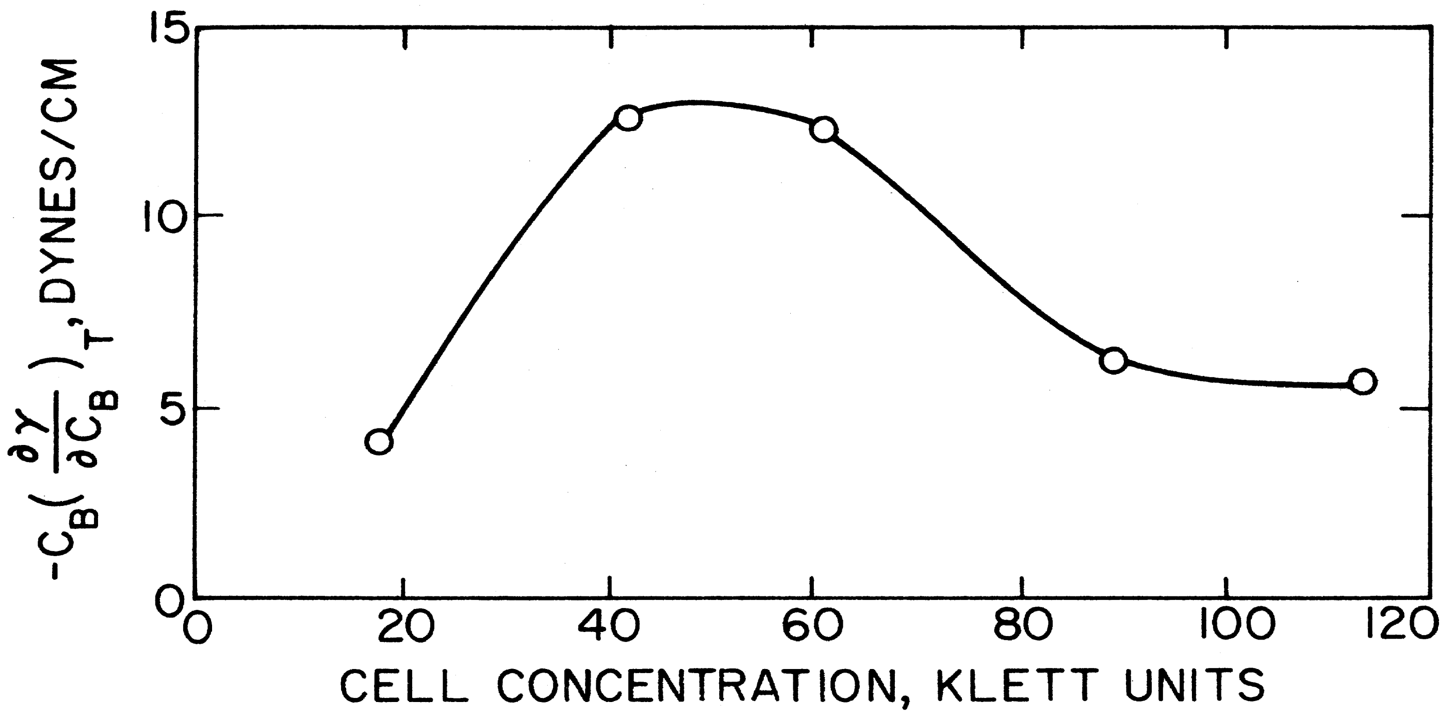


Figure 16. The Gibbs adsorption isotherm for suspensions of *Pseudomonas ovalis* in Medium B. No antifoam was present. Temperature, 25 °C; pH 7.0.

film around bubbles, the effect of pH on the rate equations for the production of gluconic acid can be considered.

Papers have appeared in the literature describing systems in which charge effects have resulted in increased reaction rates in monolayers. For example, Llopis and Davies (49) succeeded in increasing the rate of acid hydrolysis of a cholesterol formate in a film by an order of magnitude by incorporating a small amount of long chain sulphate into the film. Conversely, addition of long chain cation retarded the reaction.

The alkaline hydrolysis of octadecylacetate monolayers can be accelerated by as much as seventeen times by incorporating a little $C_{18}H_{37}N(CH_3)_3^+$ into the monolayers (22).

In the case of the gluconic acid fermentation by P. ovalis, there are many reactions occurring simultaneously, and all of them are pH dependent. The intermediate gluconolactone is hydrolyzed non-enzymatically to gluconic acid. The rate of hydrolysis decreases as the pH is lowered. Since the bacteria are negatively charged, the pH in the surface film will be lowered. The rate of hydrolysis of lactone will thus be decreased in the surface film. Changes in the rate of hydrolysis of lactone in the film therefore cannot be responsible for the accelerated fermentation rate in the presence of bubbles.

Actually, according to the kinetic model developed in this chapter, the rate of hydrolysis is not the controlling reaction in the fermentation. This is consistent with the theoretical expectation that the rate of lactone hydrolysis will be decreased in the surface film. If the lactone hydrolysis rate were controlling, then it would

be expected that increasing the surface area would decrease the overall fermentation rate. It has been shown experimentally in this thesis that increasing the interfacial area increases the fermentation rate.

It is hypothesized that the fermentation rate increases with an increase in interfacial area due to pH effects in the surface film. If this is to be accepted then the effect must be due to an increase in the net rate of formation of the lactone. This net rate is the difference in the rate of formation of the lactone from glucose, and the rate of formation of glucose from the lactone. In the steady state the difference is necessarily equal to the rate of hydrolysis of the lactone to gluconic acid.

The phenomenological effect of an increased net rate of formation of lactone will be an increased concentration of gluconolactone in the steady state. This is in fact what was observed experimentally. An increase in the interfacial area, brought about by an increase in the agitation rate, resulted in an increase in the lactone concentration at steady state.

An increase in the net rate of lactone formation will be brought about whenever there is an increase in the difference between the rate of formation of lactone from glucose and the rate of formation of glucose from lactone. In the formulation of equations from the kinetic model, the lactone concentration at steady state was assumed to be constant. This implies that $d[L]/dt$ is zero. The equation for $d[L]/dt$ can be written as follows:

$$\frac{d[L]}{dt} = k_3[ES][O_2]^{\frac{1}{2}} - k_4[E][L] - k_5[L] \qquad (48)$$

The last term in this equation represents the rate of hydrolysis of the lactone to gluconic acid. The first two terms on the right therefore represent the net rate of formation of lactone. Thus

$$\left.\frac{d[L]}{dt}\right|_{Net} = k_3[ES][O_2]^{\frac{1}{2}} - k_4[E][L] \tag{49}$$

Substituting for (ES) and (E) in terms of $(E)_o$

$$\left.\frac{d[L]}{dt}\right|_{Net} = \frac{k_1k_3[S]\,[O_2]^{\frac{1}{2}} - k_2k_4[L]}{k_1[S] + k_2 + k_3[O_2]^{\frac{1}{2}} + k_4[L]}\,[E]_o \tag{50}$$

In the steady state the net rate of formation of glucono-lactone is equal to the rate of acid formation. In this thesis, measurements were reported of steady state fermentation rates and lactone concentrations as a function of pH. Referring to Figure (12), it can be seen that there was an optimum pH for acid production. The maximum steady rates occurred at pH 7.3 to 7.5. As the pH is lowered from the optimun, the steady state acid production rate decreases monatomically. The steady state lactone concentration becomes progressively higher as the pH is lowered from the optimum. Thus if the pH were to be lowered from 7.0, the steady state acid production rate would drop, while the lactone concentration would rise.

However, the phenomenon under examination is a transient, not a steady state, effect. Two factors contribute to this. First, the air bubbles passing through the broth have short residence times, relative to the overall length of the fermentation. Furthermore, even during the relatively short life of a bubble, the liquid surrounding the bubble is continually being renewed due to viscous drag

effects. Thus, the time during which a given cell will be in the surface film around the bubbles will generally be somewhat less than the average residence time of those bubbles. From Equations (48) and (49) it can be seen that

$$\left.\frac{d[L]}{dt}\right|_{Net} = \frac{d[L]}{dt} + \frac{d[A]}{dt} \qquad (51)$$

In the steady state $d[L]/dt$ is zero. Consider, however, what will happen when a cell is brought into the surface film around a bubble. The presence of the cell in the film will contribute to a drop in the pH by an undetermined amount. The cell is effectively subjected to a disturbance or perturbation from its environment in the steady state at pH 7 to a non-steady state at a lower pH. The net rate of lactone production in the film will then be given by Equation (51).

It is not known how much the pH will drop in the bubble surface film. In addition, only a small portion of the cells in the broth will be subjected to this lower pH at any given moment. However, it is possible to produce a disturbance in a bulk fermentation which might be expected to approximate on a larger scale the situation which obtains in the surface film. The experiment measuring the response of the fermentation system to a step change in pH produced just such a disturbance. Referring to Figure (13), in this experiment a steady state was achieved at pH 7.0, and then the pH was abruptly changed to 6.7. The drop of 0.3 pH unit was entirely arbitrary. As was stated before, when the pH was first lowered to 6.7, the rate of acid production abruptly dropped. This occurred because the lactone hydrolysis constant is lower at pH 6.7 than it is at

pH 7.0. The rate of acid production just after the pH was lowered was estimated for the experiment of Figure (13) as the product of the rate constant for lactone hydrolysis at pH 6.7 and the lactone concentration at pH 7.0 just prior to lowering the pH. The rate constant at pH 6.7 was determined from the steady state acid production rate and lactone concentration at pH 6.7. The steady state acid production rate at pH 6.7 was 2.93 meq/l/hr. The corresponding lactone concentration was 7.25 mmol/l (or meq/l). The lactone hydrolysis rate constant at pH 6.7 was therefore $2.93/7.25 = 0.405\ hr^{-1}$.

The steady state lactone concentration at pH 7.0 just prior to lowering the pH was 6.23 mmol/l. The rate of acid production just after the pH was lowered to 6.7 was therefore $0.405 \times 6.23 = 2.52$ meq/l/hr. The rate of acid production as measured by the rate of alkali addition was only 1.76 meq/l/hr. However, the pH change was not instantaneous, and a certain amount of acid was produced during the interval. No alkali was added to neutralize this portion of acid. Therefore the larger figure of 2.52 meq/l/hr is probably more accurate. This figure is therefore to be used for $d[A]/dt$ in Equation (51).

The rate of change of the lactone concentration after the pH was lowered was determined from the slope of the curve of lactone concentration versus time in Figure (13). This rate, $d[L]/dt$, was 1.76 mmol/l/hr.

The net rate of lactone production just after the pH was changed from 7.0 to 6.7 is therefore given by

$$d[L]/dt\Big|_{Net} = d[L]/dt + d[A]/dt = 1.76 + 2.52$$

$$= 4.28 \text{ meq/l/hr.}$$

The net rate of lactone production at pH 7.0 at steady state was equal to the rate of acid production, about 3.96 meq/l/hr.

The net rate of lactone formation just after the pH was lowered to 6.7 was therefore higher than the steady state rate at pH 7.0. This was true even though the steady state rate at pH 6.7 was lower than the steady state rate at pH 7.0.

These findings are not necessarily contradictory. The rate of formation of lactone from glucose is a function of the concentration of glucose, and of the kinetic rate constant. The glucose was present in large excess, and its concentration could be considered constant. The new rate of formation of lactone from glucose at pH 6.7 was therefore established immediately. The rate of the back reaction, the formation of glucose from lactone, depends on the kinetic rate constant and the concentration of lactone. The rate of this back reaction, therefore, will not be a maximum until the lactone concentration rises to a new value. The back reaction therefore lags the forward reaction during the transient period.

As was stated before, it is not known how much the pH dropped in the surface film during the experiments measuring the effect of agitation on the fermentation rate. However, the experiment measuring the transient response of the fermentation system as a whole to a step change in pH shows how it is possible to increase the net rate of lactone formation briefly. A similar phenomenon might be occurring in the surface film around the bubbles during steady state experiments.

The overall process might be as follows. Consider an element

of the bulk fluid in the fermentor at a steady state at pH 7.0. When this element is brought into the vicinity of a bubble surface, the pH in the element will drop. In addition, a certain number of excess cells will be brought into the element, causing a further drop in pH. The fluid element will no longer be in the steady state, and the net rate of lactone formation will be higher than when the element was in the bulk fluid. The result will be a rise in the lactone concentration in the element.

Eventually the fluid element will be returned to the bulk fluid. The lactone concentration in the element will be higher when the element leaves the bubble surface than when it was first brought to that surface. The net result is to raise the concentration of lactone in the bulk fluid. Since the acid production rate is proportional to the lactone concentration, it will also be raised. The larger the bubble surface area, the larger will be the fraction of cells at the lower pH at a given moment. Thus the larger the surface area, the higher the acid production rate.

It has been shown that the net rate of lactone formation can be increased momentarily in the gluconic acid fermentation by abruptly lowering the pH of the fermentation broth. It has been hypothesized that near the gas-liquid interface around the air bubbles in the fermentor, the pH will be lowered by an undetermined amount. It is therefore plausible that the net rate of lactone formation in the vicinity of the gas-liquid interface will be higher than the rate in the bulk liquid. If such a phenomenon does occur, then it follows that increasing the gas-liquid interfacial area in the fermentor will

result in an increase in the net rate of lactone formation. This will cause an increase in the bulk lactone concentration, and the rate of formation of gluconic acid will therefore be increased. In experiments reported in this thesis, the bulk lactone concentration and the gluconic acid production rate both increased when the gas-liquid interfacial area was increased by raising the agitation rate.

In another series of experiments reported in this thesis, the effect of the gas-liquid interfacial area on certain aspects of the fermentation were measured independently of oxygen transfer. In these experiments a steady state was established and then all of the oxygen was displaced from the fermentor with nitrogen gas. Any dissolved oxygen in the broth was either stripped away or quickly metabolized. Thus in a relatively short time all molecular oxygen was removed from the system. The lactone concentration at this point was still high. The rate of disappearance of the lactone, and the rate of production of gluconic acid, after all molecular oxygen had been removed, were measured for two cases: 1) with nitrogen bubbled through the broth at 1.0 VVM, the same rate as air had previously been bubbled through, and 2) with nitrogen passed only through the head space of the fermentor. The difference between the two cases was that in the former case the gas-liquid interfacial area was approximately the same as during the steady state with oxygen present, while in the latter case the interfacial area was negligibly small.

If the hypothesized lowering of the pH in the vicinity of the gas-liquid interface actually occurred, then it would be expected that the presence of inert gas bubbles would decrease the rate of

lactone hydrolysis to gluconic acid. This was found to be the case experimentally. The apparent kinetic rate constant for the production of gluconic acid was smaller in the presence of nitrogen bubbles, in every instance studied except one.

The apparent kinetic rate constant for the overall rate of disappearance of the lactone in the presence of nitrogen bubbles was higher than the rate constant when nitrogen was passed through the head space of the fermentor in every case. Moreover, the apparent rate constants for lactone disappearance were larger than the apparent rate constants for acid production. Thus not all of the lactone present in the broth was hydrolyzed to gluconic acid. The chemical reactions are all reversible except for the hydrolysis of the lactone to gluconic acid. Therefore, it is reasonable to assume that any lactone not hydrolyzed to gluconic acid was used to form glucose. The difference between the rate constants calculated for the overall rate of lactone disappearance and the rate constants for acid production is a measure of the rate of formation of glucose from the lactone. This difference was larger in the presence of nitrogen bubbles than it was when nitrogen was passed through the head space of the fermentor. Thus it might be inferred that the back reaction was faster in the surface phase than it was in the bulk phase. This does not necessarily contradict the hypothesized effect. However, in order for the hypothesized effect to obtain under such a condition it would be necessary for the forward reaction to increase even more. In such a case Equation (37) would no longer be valid.

Actually, the numbers obtained from the data are not sufficiently accurate to allow any conclusions to be drawn concerning changes in the relative rates. The important finding is that there is a significant difference between the rate of lactone disappearance and the rate of gluconic acid production under the anaerobic conditions of these experiments. This demonstrates indirectly that the enzymatic reactions are reversible.

Finally, some discussion of the experiments on the effects of viscosity on the fermentation rate is warranted. It was found experimentally that increasing the viscosity of the fermentation broth with Natrosol 250H by an amount sufficient to reduce the specific oxygen transfer coefficient $k_L^o a$ by about one half had no significant effect on the rate of acid production. The amount of viscosity additive used was not sufficient to cause the dissolved oxygen level to fall below the critical. Therefore, according to current concepts, as outlined in the classification scheme of Astarita (7), increasing the viscosity by this amount should have no effect on the fermentation rate, as was found experimentally.

However, one of the effects of Natrosol 250H is to increase the interfacial area obtained in the fermentor at a given aeration and agitation rate. If the surface effects hypothesized in this section do occur, then it would seem that increasing the interfacial area should increase the rate of acid production at steady state. Actually, the question is somewhat more complex. The magnitude of the hypothesized surface effect should depend somewhat on the rate at which fresh bulk fluid is brought into the surface film. Although

addition of Natrosol 250H to the fermentation broth probably did increase the interfacial area, it also reduced the circulation rate of liquid around the bubble. This reduced circulation might have reduced the pH effects in the surface phase sufficiently to offset the effect of an increased interfacial area.

The hypothesis presented here is extremely tentative, and much work can be suggested for further investigation. For instance, it should be fruitful to determine if the increase in the acid production rate brought about by increased agitation can be demonstrated at bulk pH values other than 7.0. The optimum pH for acid production was about 7.3. It would be interesting to determine if the phenomenon will occur at a pH higher than the optimum.

SUMMARY

Aerobic fermentations are usually found to proceed at a rate that is independent of the dissolved oxygen activity in the fermentation broth, provided the activity is above the "critical" value which is characteristic of the organism involved. In this thesis, it has been shown theoretically that such fermentations are controlled by chemical reaction rates.

One consequence of the theory of the critical oxygen concentration is that increasing the agitation intensity should have no effect on the rate of the fermentation. The conversion of glucose to gluconic acid by resting cells of *Pseudomonas ovalis* in a nitrogen-free medium has been shown to be a fermentation for which a critical dissolved oxygen concentration exists. The rate of gluconic acid production in the steady state was independent of the dissolved oxygen activity when that activity was above the critical level. Nevertheless, it was found that increasing the agitation rate increased the rate of acid production in the steady state. This was true even though the dissolved oxygen activity was always above the critical.

In the conversion of glucose to gluconic acid by *P. ovalis* the cells dehydrogenate glucose to produce a gluconolactone, which is then nonenzymatically hydrolyzed to gluconic acid. When the steady state rate of production of gluconic acid was increased by increasing the agitation rate, it was found that the concentration of gluconolactone in the broth was also increased. Since the acid production rate was proportional to the gluconolactone concentration, the increase in the latter was responsible for the increased rate of gluconic acid production.

A kinetic model of the fermentation was developed which predicts that the rate of production of gluconic acid in the steady state will be proportional to the lactone concentration; it will also be independent of the dissolved oxygen concentration when the latter is above a certain critical level.

It was hypothesized that the increase in the rate of production of gluconic acid was caused by an increase in the net rate of lactone formation. The principal consequence of an increase in the agitation rate in the fermentation was an increase in the gas-liquid interfacial area. Therefore, it was proposed that the net rate of formation of lactone was higher in the film around a gas bubble than in the bulk liquid. Experiments showed that the rates of disappearance of lactone from the broth in the absence of molecular oxygen were altered in a complex manner by the presence of bubbles of pure nitrogen in the broth.

A mechanism was proposed to explain the hypothesized increase in the net rate of lactone formation at the gas-liquid interface. It was proposed that cells adsorb on the surfaces of bubbles. Since the cells are negatively charged, they attract protons to the interface. This caused the pH to be lowered in the surface film around the bubbles. It was proposed that the net rate of lactone formation at the lower pH existing in the liquid around the bubbles was higher than the net rate in the bulk fluid, during the relatively short life of the bubbles.

The response of the fermentation system to a sudden reduction of the pH was evaluated. It was found that the net rate of lactone

formation immediately after the pH was reduced was higher than it had been just previous to this event. This experiment was proposed as an approximation, on a larger scale, of the phenomenon hypothesized for the surface film around bubbles.

A small amount of a hydroxyethyl ether of cellulose was added to the broth in a fermentation which was operating in the steady state. The cellulose ether increased the viscosity of the broth to approximately 200 cp. This was sufficient to lower the oxygen transfer coefficient from air to the broth by about 50 per cent. The addition of the cellulose ether had no effect on the rate of production of gluconic acid.

APPENDIX A

Derivation of the Gibbs Equation for Adsorption at a Gas-Liquid Interface.

Gibbs (33) derived an equation relating surface adsorption to interfacial tension, from thermodynamic considerations. The derivation of this equation given here follows closely the derivation outlined by Davies and Rideal (23).

Consider the gas-liquid interface to be situated along the plane CC' in Figure (17). The actual interface is not well defined, and it will have a finite thickness. Therefore, it will be maintained that the surface is somewhere between two planes AA' and BB'. These planes are defined such that at and near AA' the properties of the system are identical with those of the liquid phase, and at and near BB' the properties are identical with those of the gas phase. In this surface phase the total internal energy is given by

$$U^{\sigma} = TS^{\sigma} - PV^{\sigma} + \gamma A + \sum \mu n^{\sigma} \quad (52)$$

where γ is the interfacial tension, A is the total area, μ is the chemical potential of any given species, and n is the number of molecules of that component. If we subtract from this equation the corresponding quantities which would have obtained had the bulk phases been unchanged up to the plane CC', the result is

$$U_s = TS_s + \gamma A + \sum \mu n_s \quad (53)$$

where the subscript s refers to the excess quantity in the real surface. The temperature, pressure, and chemical potential are constant throughout the system. The term in γA does not enter the equations

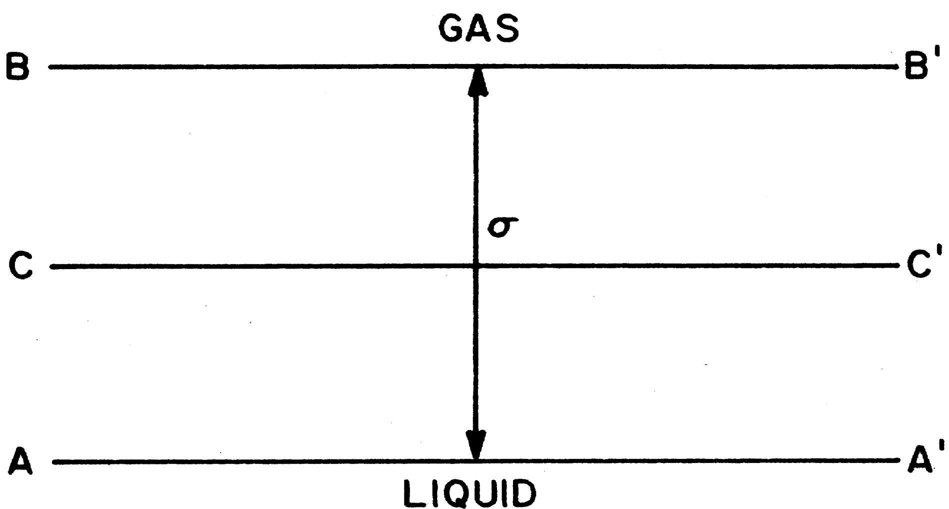


Figure 17. Hypothetical gas-liquid interface for the derivation of the Gibbs adsorption isotherm.

for the bulk phases. The volumes between AC and CB add up to V^{σ}. These factors were taken into account in writing Equation (53).

Equation (53) can be differentiated, leading to

$$dU_s = TdS_s + S_s dT + \gamma dA + Ad\gamma + \sum \mu dn_s + \sum n_s d\mu \qquad (54)$$

For a small, reversible change in the surface phase very close to equilibrium, the change in the internal energy is given by

$$dU^{\sigma} = TdS^{\sigma} - PdV^{\sigma} + \gamma dA + \sum \mu dn^{\sigma} \qquad (55)$$

Proceeding as before, subtracting from Equation (55) the corresponding quantities which would have contributed to U had the bulk phases been unchanged up to the plane CC', we have

$$dU_s = TdS_s + \gamma dA + \sum \mu dn_s \qquad (56)$$

Eliminating dU_s between Equation (54) and Equation (56) we have

$$S_s dT + Ad\gamma = -\sum n_s d\mu \qquad (57)$$

At constant temperature

$$\partial\gamma \Big|_T = -\sum n \partial\mu \Big|_T \qquad (58)$$

where $n = n_s/A$. Equation (58) is the Gibbs adsorption isotherm for the adsorption of surface active substances at a gas-liquid interface.

It follows from Equation (58) that if the interfacial tension of a fermentation broth is affected by the presence of bacteria, then adsorption of bacteria is taking place. In the case of such adsorption in the presence of excess phosphate salts, the Gibbs equation can be written

$$\partial\gamma \Big|_T = -\left[n_B \partial\mu_B + n_{K^+} \partial\mu_{K^+} + n_{PO_4^{\equiv}} \partial\mu_{PO_4^{\equiv}} \right] \Big|_T \qquad (59)$$

At equilibrium the chemical potentials in the liquid phase and in the surface phase are equal, so for each ionic species

$$\mu^{\sigma} = \mu = \mu^{o} + kT\ln C \tag{60}$$

In Equation (60) it has been assumed that the concentration of a chemical species can be substituted for its activity.

Using Equation (60), Equation (59) can be written

$$\partial\gamma\Big|_T = -kT\left[n_B \frac{\partial c_B}{c_B} + n_{K^+}\frac{\partial c_{K^+}}{c_{K^+}} + n_{PO_4^{\equiv}}\frac{\partial c_{PO_4^{\equiv}}}{c_{PO_4^{\equiv}}} \right]_T \tag{61}$$

Since the salt is present in excess, the last two terms of Equation (61) are negligible, so we have

$$-C_B\left(\frac{\partial\gamma}{\partial C_B}\right)_T = kTn \tag{62}$$

where C_B is the concentration of bacteria.

APPENDIX B

Calibration of the Klett-Summerson Photocolorimeter for the Colorimetric Determination of Gluconolactone.

Calibration of the Klett-Summerson photocolorimeter was done according to the method of Lien (47). For this calibration, 1 ml samples of gluconic acid were prepared at pH 1.5 to 2.0 in Klett-Summerson tubes. The pH was adjusted with hydrochloric acid. The samples varied in gluconic acid concentration from 0.001 M to 0.01 M, in increments of 0.001 M. The tubes were loosely covered with stainless steel caps and autoclaved at 15 pounds gage pressure for 15 minutes. The autoclaving quantitatively converted the gluconic acid to gluconolactone.

Each sample was then treated as follows. Two millimeters of hydroxylamine reagent were added to the sample. The reagent was prepared by mixing equal volumes of 4 M hydroxylamine hydrochloride and 4 N sodium hydroxide, and adjusting the pH to 8.0. The reaction mixture containing the sample and the hydroxylamine reagent was then acidified with 1 ml of 4 N hydrochloric acid. Color was formed by the addition of 1 ml of a solution of ferric chloride, containing 100 grams per liter of $FeCl_3$ in 0.1 N HCl. Photometric determinations were made with the Klett-Summerson photocolorimeter using a green, 540 mμ filter. Untreated samples were maintained at 80 to 90 ^{o}C, until just before treatment. The blank was made by treating a sample of 0.01 M gluconic acid in the manner just described, but without autoclaving.

The readings of the photocolorimeter obtained as a function of the lactone concentrations of the samples are presented in Figure (18).

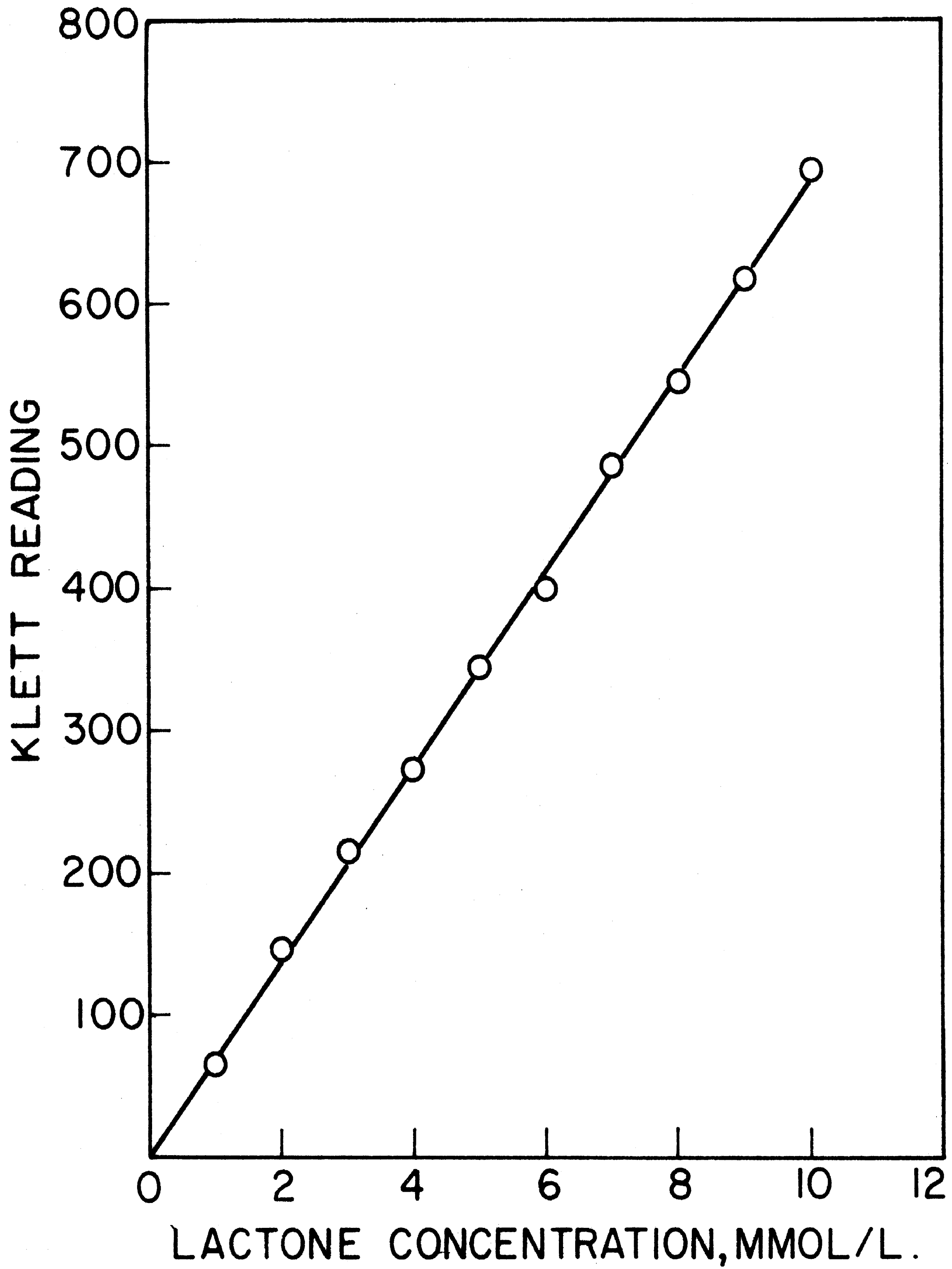


Figure 18. Calibration of the Klett-Summerson photocolorimeter at 540 mμ for the determination of gluconolactone.

APPENDIX C

<u>Sample Calculation of the Specific Physical Oxygen Absorption Coefficient.</u>

The experimental data for the calculation of oxygen absorption coefficients were obtained as continuous recorder traces of E versus t. In order to calculate the absorption coefficients, it was necessary to choose a time interval and note the oxygen electrode readings at each time. The instantaneous specific physical absorption coefficient for any given experimental conditions between times t_n and t_{n+1} could then be determined from Equation (29), after slight rearranging:

$$k_L^o a = \frac{E_{n+1} - E_n}{(t_{n+1} - t_n)(E_S - \frac{E_{n+1} + E_n}{2})}$$

For example, for the date of Figure (3) for the absorption of oxygen to Medium B at 434 RPM and 1.0 VVM, the electrode reading changed from 80.0 mv to 83.2 mv between the 0.8 minute and 0.9 minute marks. Therefore, for this time interval,

$$k_L^o a = \frac{83.2 - 80.0}{(0.9 - 0.8)\ (100.0 - \frac{83.2 + 80.0}{2})} = 1.74\ \text{min}^{-1}$$

The average coefficient was estimated by calculating $k_L^o a$ for each time interval over the entire span in Figure (3), and then calculating the average of all values of $k_L^o a$. The complete calculations for this case are presented in Table XI. The calculations for all absorption coefficients reported in this thesis were exactly similar. The exception was that no average values were determined for surface reaeration, since those coefficients were concentration dependent.

TABLE XI

CALCULATION OF THE COEFFICIENT FOR THE ABSORPTION OF OXYGEN FROM AIR TO MEDIUM B. AGITATION RATE, 434 RPM; AERATION RATE, 1.0 VVM; LIQUID VOLUME, 2 LITERS; TEMPERATURE, 25 °C.

Time min.	E, Per Cent Saturation	$\Delta E/\Delta t$ Per Cent per Minute	$E_S - E_{AVG}$ Per Cent Saturation	$k_L^o a$ min^{-1}
0.1	28.5			
0.2	40.8	123	65.35	1.88
0.3	51.0	102	54.1	1.89
0.4	59.2	82	44.9	1.83
0.5	65.8	66	37.5	1.76
0.6	71.4	56	31.4	1.78
0.7	76.0	46	26.3	1.75
0.8	80.0	40	22.0	1.82
0.9	83.2	32	18.4	1.74
1.0	85.7	25	14.55	1.72
1.1	87.9	22	13.2	1.67
1.2	89.8	19	11.15	1.71
1.3	91.4	16	9.4	1.70
1.4	92.8	14	7.9	1.77
1.5	93.9	11	6.65	1.66
1.6	94.8	9	5.65	1.59
1.7	95.6	8	4.8	1.67

Average value of $k_L^o a$ = 1.75 min^{-1}.

APPENDIX D

<u>Calculation of the Oxygen Utilization Rate from the Rate of Gluconic Acid Production.</u>

The rate of oxygen utilization, as measured by the steady state rate of gluconic acid production, can be calculated from the rate of alkali addition to the broth:

$$R_G = \frac{1}{2} \frac{N_b}{V} \frac{dv_b}{dt} \quad \text{(mmoles/l/hr)}$$

R_G is the rate of oxygen utilization, N_b is the normality of the base, and dv_b/dt is the volumetric rate of alkali addition.

For the run of Figure (10), with a 0.490 N sodium hydroxide solution, the rate of alkali addition at 390 RPM was 22.2 ml/hr. Therefore,

$$R_G = \frac{1}{2} \left(\frac{0.490}{2}\right) (22.2) = 2.72 \text{ mmoles/l/hr.}$$

To calculate the rate of oxygen utilization by the acid production rate just after aeration was stopped, the rate of alkali addition during the five-minute interval after the air supply was cut off was used for dv_b/dt. For the run of Figure (10), at 390 RPM, 2.1 ml of 0.490 N alkali were added during this five-minute interval, which was an alkali addition rate of 25.2 ml/hr. Therefore,

$$R_{GO} = \frac{1}{2} \left(\frac{0.490}{2}\right) (25.2) = 3.09 \text{ mmoles/l/hr.}$$

where R_{GO} is the rate of oxygen utilization just after aeration was stopped.

APPENDIX E

Calculation of the Oxygen Utilization Rate using the Oxygen Electrode.

In some experiments, the oxygen uptake rate was computed by measuring the rate of depletion of dissolved oxygen in the fermentation broth, after the air supply to the sparger had been cut off. The rate of oxygen utilization in such a case is proportional to the rate of change of the oxygen electrode reading. However, a small correction must first be applied to the rate of change of the electrode reading. This correction accounts for the small amount of leakage of oxygen which will occur through the surface of the liquid during removal of dissolved oxygen from the broth by the cells.

Such corrections were calculated in the following manner. For the run of Figure (10), at 390 RPM, the electrode reading changed from 58.5 mv to 54.4 mv during the time interval from 0.4 minutes to 0.6 minutes of the dissolved oxygen depletion curve. This was a rate of change of 20.5 mv/min., at an average dissolved oxygen concentration of 56.45 mv, or per cent saturation. The average driving force for surface absorption during the interval was 100 - 56.45 = 43.55 mv. Regardless of the prior agitation rate, the agitation rate was always changed to 121 RPM during the depletion rate measurements, in order to minimize surface reaeration. A curve for surface reaeration similar to Figure (9) was therefore obtained at 121 RPM. The data for such a curve at 121 RPM are presented in Table XII. From this table, the rate of surface reaeration at a driving force of 43.55 mv was approximately 0.6 mv/min. The total rate of oxygen uptake by the cells during the time interval from 0.4 to 0.6 minutes was therefore

TABLE XII

THE RATE OF SURFACE REAERATION OF MEDIUM B WHICH HAD BEEN STRIPPED WITH NITROGEN. LIQUID VOLUME, 2 LITERS; AERATION RATE, 0 VVM; AGITATION RATE, 121 RPM; TEMPERATURE, 25 °C.

$E_S - E_{AVG}$, Per Cent Saturation	$\Delta E/\Delta t$, Per Cent per Minute
24.05	0.31
27.45	0.37
30.4	0.44
32.6	0.44
35.8	0.52
37.45	0.54
40.3	0.60
43.35	0.62
46.65	0.70
50.3	0.76
54.2	0.8
57.1	0.9
58.95	0.95
60.85	0.95
62.85	1.05
64.95	1.05
67.05	1.05
69.2	1.1
71.55	1.25
74.1	1.3
76.7	1.3
79.4	1.4
82.15	1.35
85.0	1.5
88.05	1.55
91.2	1.6

$$\frac{dE}{dt} = 20.5 + 0.6 = 21.1 \quad mv/min.$$

Finally, dE/dt was converted to appropriate units:

$$R_E = (\frac{60}{32}) \frac{C_s}{(E_s - E_o)} \frac{dE}{dt} = (\frac{60}{32})(\frac{6.8}{100})(21.1)$$

$$= 2.69 \text{ mmoles/l/hr.}$$

R_E is the rate of oxygen utilization measured with the oxygen electrode.

Calculations were carried out for the entire oxygen depletion curve, as shown in Table XIII. The actual value of dE/dt used to calculate R_E at 390 RPM for Figure (10) was estimated from these data as about 21 mv/min.

TABLE XIII

CALCULATION OF THE RATE OF OXYGEN UTILIZATION FROM THE RATE OF DEPLETION OF DISSOLVED OXYGEN IN THE MEDIUM AFTER THE AIR SUPPLY HAD BEEN CUT OFF. LIQUID VOLUME, 2 LITERS; AGITATION RATE, 390 RPM; pH, 7.0; TEMPERATURE, 25 °C; CELL CONCENTRATION, 100 KLETT UNITS.

Time, min.	E, Per Cent Saturation	$E_S - E_{AVG}$ Per Cent Saturation	$(\Delta E/\Delta t)$ Per Cent per Minute	$(\Delta E/\Delta t)_L$ Per Cent per Minute	$(\Delta E/\Delta t)_T$ Per Cent per Minute
0	66.9				
0.2	62.5	35.3	22.0	0.5	22.5
0.4	58.5	39.5	20.0	0.6	20.6
0.6	54.4	43.55	20.5	0.6	21.1
0.8	50.3	47.65	20.5	0.7	21.2
1.0	46.3	51.7	20.0	0.8	20.8
1.2	42.1	55.8	21.0	0.85	21.85
1.4	38.1	59.9	20.0	0.95	20.95
1.6	34.1	63.9	20.0	1.05	21.05
1.8	30.5	67.7	18.0	1.05	19.05
2.0	26.8	71.35	18.5	1.2	19.7
2.2	23.1	74.05	18.5	1.3	19.8
2.4	19.5	78.7	18.0	1.35	19.35
2.6	16.0	82.25	17.5	1.35	18.85
2.8	12.7	85.65	16.5	1.5	18.0
3.0	9.4	88.95	16.5	1.55	18.05

APPENDIX F

Calculation of the Kinetic Constants for Lactone Disappearance in the Absence of Molecular Oxygen.

Measurements of the kinetic constants for the disappearance of lactone, as presented in Table X, were calculated from the equations

$$-\frac{d[L]}{dt} = K_9[L]$$

$$\frac{d[A]}{dt} = k_5[L]$$

$$R_G = k_5^{O_2}[L]$$

The unprimed constants apply to the cases for which nitrogen was passed through the head space of the fermentor. Primed constants, for the cases in which nitrogen was sparged through the fermentor, were calculated from equations identical in form to the above.

The actual calculations were carried out using differences rather than derivatives:

$$K_9 = -(\Delta L/\Delta t)/L_{AVG}$$

$$k_5 = (\Delta A/\Delta t)/L_{AVG}$$

For the calculations of K_9 or K_9', it was dimensionally sound to use the Klett readings obtained, rather than the actual lactone concentrations. This could not be done, however, in calculating k_5, k_5', or $k_5^{O_2}$.

For the data of Figure (14) at 310 RPM, with nitrogen passed through the head space of the fermentor, during the time interval from 20 minutes to 24 minutes the lactone concentration changed from a Klett reading of 265 to a reading of 245. This was a rate of change

of 5 units/min. The average Klett reading during the interval was 255. Therefore, for this interval

$$K_9 = 5/255 = 1.96 \times 10^{-2} \text{min}^{-1}$$

During this same interval, 0.7 ml of 0.497 N sodium hydroxide solution were added, so

$$\frac{d[A]}{dt} = \frac{1}{2}(0.497)(\frac{0.7}{4}) = 4.35 \times 10^{-2} \text{ meq/l/min}$$

The average lactone concentration during the interval was 255 Klett units. From Figure (18), this corresponds to a lactone concentration of 3.7 meq/l. Therefore, for this interval

$$k_5 = 4.35 \times 10^{-2}/3.7 = 1.18 \times 10^{-2} \text{min.}^{-1}$$

The complete calculations for this case are presented in Table XIV. Values of the constants entered in Table X are the average values from Table XIV.

During the steady state, prior to the introduction of nitrogen into the fermentor, alkali was being added to the fermentor at a rate of 0.37 ml/min., so

$$R_G = \frac{1}{2}(0.37)(0.497) = 9.2 \times 10^{-2} \text{meq/l/min}$$

The steady state lactone concentration was 415 Klett units, or 6.02 meq/l. Therefore

$$k_5^{O_2} = 9.2 \times 10^{-2}/6.02 = 1.53 \text{ min.}^{-1}$$

TABLE XIV

CALCULATION OF THE RATE OF ACID PRODUCTION AND THE RATE OF DISAPPEARANCE OF GLUCONOLACTONE FROM A FERMENTATION BROTH IN THE GLUCONIC ACID FERMENTATION OF <u>Pseudomonas</u> <u>ovalis</u> AFTER THE AIR SUPPLY HAD BEEN CUT OFF. THE HEAD SPACE OF THE FERMENTOR WAS FILLED WITH NITROGEN GAS. LIQUID VOLUME, 2 LITERS; AGITATION RATE, 310 RPM; TEMPERATURE, 25 °C; CELL CONCENTRATION, 100 KLETT UNITS.

Time, min.	(1) $\Delta A/\Delta t$ x 10^2 meq/l/min	Lactone Conc., Klett Units	(2) Average Lactone Conc., Klett Units	(3) Average Lactone Conc., mmol/l	(4) $-\Delta L/\Delta t$ Klett Units per min.	(4)/(2) K_9 x 10^2 min^{-1}	(1)/(3) k_5 x 10^2 min^{-1}
0	-	375	-	-	-	-	-
4	7.45	345	360	5.22	-	-	1.43
8	5.60	325	335	4.86	5.0	1.49	1.15
12	5.60	295	310	4.49	7.5	2.42	1.24
16	2.49	270	282.5	4.10	6.25	2.21	0.61
20	6.22	260	265	3.84	2.5	0.94	1.62
24	3.73	247	253.5	3.68	3.25	1.28	1.01
28	1.87	235	241	3.50	3.0	1.24	0.53
32	4.35	220	227.5	3.30	3.75	1.65	1.32

Average value of K_9 = 1.60 min^{-1}.

Average value of k_5 = 1.11 min^{-1}.

APPENDIX G

Nomenclature

a	Gas-liquid interfacial area per unit volume of liquid
a_i^b	Activity of species i in the bulk phase
a_i^σ	Activity of species i in the surface phase
a_m	Total cell interfacial area per unit volume of liquid
a_W	Activity of water
A	Total gas-liquid interfacial area
$[A]$	Concentration of gluconate
A_i	Molar surface area of species i
$\bar{A}_i$	Partial molar surface area of species i
B_i	Reacting species i
C	Dissolved oxygen concentration
C_s	Dissolved oxygen concentration at saturation
C^*	Dissolved oxygen concentration at the gas-liquid interface
C_e	Dissolved oxygen concentration at chemical equilibrium
C_{cr}	Critical dissolved oxygen concentration
C_B	Concentration of bacteria
d	Diameter of cells
D_L	Diffusivity of oxygen in the fermentation medium
E	Reading of the oxygen analyzer at a dissolved oxygen concentration of C
E_o	Reading of the oxygen analyzer in a solution devoid of molecular oxygen
E_s	Reading of the oxygen analyzer in a solution saturated with molecular oxygen
$[E]$	Concentration of free enzyme
$[E]_o$	Total enzyme concentration, equal to $[E] + [ES]$

$[ES]$	Concentration of enzyme-glucose complex
G	Outlet gas flow rate from the fermentor
G_o	Inlet gas flow rate to the fermentor
H	Henry's law coefficient
k	Boltzmann constant
k_c	Specific reaction rate constant, defined by $r = k_c a_m C_{cr}$
k_G	Gas phase oxygen transfer coefficient
$k_1, \ldots k_5$	Kinetic rate constants in the reaction model for the production of gluconic acid
k_5	First order rate constant for acid production with nitrogen passed through the head space of the fermentor
k_5'	First order rate constant for acid production with nitrogen sparged through the fermentation medium
k_5^{O2}	First order rate constant for acid production in the steady state with a constant lactone concentration
k_L	Coefficient for absorption of oxygen with chemical reaction
k_L^o	Coefficient for physical absorption of oxygen
k_m	Coefficient for oxygen transfer from the bulk liquid to cells
$k_L a$	Specific oxygen transfer coefficient for chemical absorption
$k_L^o a$	Specific oxygen transfer coefficient for physical absorption
$(k_L^o a)_o$	Specific oxygen transfer coefficient for physical absorption through the top surface of the liquid with no sparging
K^b	Equilibrium constant in the bulk phase
K^σ	Equilibrium constant in the surface phase
K_G	Overall gas phase oxygen transfer coefficient
K_M	Michaelis constant
$K_1, \ldots, K_9$	Lumped kinetic coefficients in the reaction model for the production of gluconic acid

K_9	First order rate coefficient for the disappearance of lactone with nitrogen passed through the head space of the fermentor
K_9'	First order rate coefficient for the disappearance of lactone with nitrogen sparged through the fermentation medium
$[L]$	Concentration of gluconolactone
m	Constant of proportionality in converting oxygen analyzer readings to concentration units
n	Excess concentration in the surface phase per unit interfacial area, equal to n_s/A
n_s	Excess concentration in the surface phase
N_{Sh}	Sherwood number, $k_m d/D_L$
N	Number of time increments in the determination of $k_L^o a$
$[O_2]$	Dissolved oxygen concentration
P	Pressure
q	Instantaneous oxygen absorption rate
r	Volumetric rate of oxygen consumption by the cells
R	Universal gas constant
R_E	Rate of oxygen utilization measured with the oxygen electrode
R_G	Rate of oxygen utilization determined from the steady state acid production rate
R_{GO}	Rate of oxygen utilization determined from the acid production rate just after the air supply was cut off
S	Entropy of bulk phase
S_s	Excess entropy of surface phase
S^σ	Entropy of surface phase
$[S]$	Concentration of glucose
t	Time
t_D	Equivalent diffusion time, defined in Equation (16)

t_r	Reaction time, defined in Equation (17)
T	Absolute temperature
U	Internal energy of bulk phase
U_s	Excess internal energy of surface phase
U^{σ}	Internal energy of surface phase
V	Volume of liquid medium
V	Volume of surface phase
$[W]$	"Concentration" of water, assumed equal to a_W
y	Mole fraction of oxygen in the exhaust gas from the fermentor
y_o	Mole fraction of oxygen in the inlet gas to the fermentor
γ	Interfacial tension of a mixture against air
γ_i	Interfacial tension of pure species i against air
ϵ	Electronic charge in e.s.u.
ψ	Electrostatic potential at the gas-liquid interface due to a net negative charge in the surface film
μ	Chemical potential
μ°	Chemical potential in the standard state
μ^{σ}	Chemical potential in the surface phase
ν_i	Stoichiometric coefficient of species i

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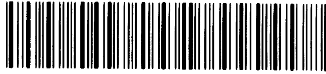
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