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Bicarbonate Requirement for Elimination of the Lag Period of *Hydrogenomonas eutropha*

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Carbon dioxide and oxygen concentrations have a profound effect on the lag period of chemoautotrophically grown *Hydrogenomonas eutropha*. Minimum lag periods and high growth rates were obtained in shaken flask cultures with a prepared gas mixture containing 70% H_2, 20% O_2, and 10% CO_2. However, excessively long lag periods resulted when the same gas mixture was sparged through the culture. The lag period was shortened in sparged cultures by decreasing both the pO_2 and the pCO_2, indicating that gas medium equilibration had not occurred in shaken cultures. The lag period was completely eliminated at certain concentrations of O_2 and CO_2. The optimum pO_2 was 0.05 atm, but the optimum pCO_2 varied according to the *p*H of the medium and physiological age of the inoculum. At *p*H 6.4, the pCO_2 required to obtain immediate growth of exponential, postexponential, and stationary phase inocula at equal specific rates was 0.02, 0.05, and 0.16 atm, respectively. With each 0.3-unit increase in the *p*H of the medium, a 50% decrease in the CO_2 concentration was needed to permit growth to occur at the same rate. The pCO_2 changes required to compensate for the *p*H changes of the medium had the net effect of maintaining a constant bicarbonate ion concentration. Initial growth of *H. eutropha* was therefore indirectly related to pCO_2 and directly dependent upon a constant bicarbonate ion concentration.

Species of *Hydrogenomonas* are partially characterized by their ability to grow in certain ranges of oxygen concentration (*Bergey's Manual*, 7th ed.; 3, 4), the extent of growth being the primary determinant. Critical data on the effect of the partial pressure of oxygen on exponential growth rates have been presented by Schlegel and Lafferty (9) and Bongers (1). Although CO_2 is a primary substrate for autotrophic growth, there are little data on the effect of CO_2 concentration on cell growth. Until recently (1), only the effects of gross variations in pCO_2 were reported. The idea that cell growth would respond to small changes in CO_2 concentration seems to have been neglected.

It is tacitly assumed that a nutritionally complete medium which supports exponential growth of cells also provides the most favorable environment for initial growth of an inoculum, even if the inoculum consists of physiologically old cells. Data presented in this paper with *Hydrogenomonas eutropha* show that inocula from cultures of different physiological ages have different initial growth requirements for CO_2. When the appropriate CO_2 concentration was provided, exponential growth began immediately at maximum specific rates. The optimum pCO_2 for immediate growth varied with the *p*H of the medium. These data showed that bicarbonate was the species of CO_2 used by the cells. Need for the unique pCO_2 changed after one to two cell generations, and thereafter all cultures had a common CO_2 requirement.

MATERIALS AND METHODS

H. eutropha inocula were prepared as liquid cultures grown chemoautotrophically at 31 C on a rotary shaker in 500-ml baffled flasks containing 100 ml of salts medium (6, 7). The premixed gas atmosphere contained 70% hydrogen, 20% oxygen, and 10% carbon dioxide. After incubation, a special rubber stopper (7), by which the flask was connected to the gas manifold, was replaced with a sterile cotton plug. Stock cultures could be stored for a month at room temperature without significant loss of viability. Culture purity was determined by microscopic examination and by plating on Trypticase soy agar. Viable cell counts determined on Trypticase soy agar in air or on mineral salts medium (Noble agar base) in the gas mixture were the same; reliable cell counts therefore could be made under heterotrophic conditions (11).

In experiments in which growth was studied as a function of the partial pressures of oxygen and carbon

dioxide, a 2.5-liter fermentor containing 1 liter of medium was used. The fermentor consisted of a cylindrical glass vessel containing individual spargers for H_2, O_2, and CO_2. Near the base of the vessel was a port fitted with a serum bottle stopper through which a combination *p*H electrode was inserted. Culture samples were also withdrawn by syringe through the serum bottle stopper. Gas dispersion was accomplished by a double-bladed impeller driven by a "Lightnin" (Fisher Scientific Co., Fair Lawn, N.J.) air-driven motor mounted on the metal head plate. The impeller bearing in the head plate was a Teflon sleeve with an "O" ring to provide a positive seal (Chesapeake Stirrer, Van Dyk Research Corp., Hackettstown, N.J.). The head plate was also fitted with an addition port and a gas exhaust line.

Gas proportions were calculated from relative gas flow rates which were regulated by individual flow meters. Each gas entering the fermentor was filtered through sterile glass wool; excess gas was vented through the head plate and a filter before being discharged into an air exhaust duct where dilution removed the explosive hazard. Equilibration between the gas and liquid phases was established by preflushing the medium with the gases for 30 min prior to inoculation. With few exceptions the minor gas component in a given mixture had a flow rate that exceeded 100 ml per min.

Growth was measured by the increase in optical density (OD) at 660 nm (Gilford spectrophotometer, Gilford Instrument Laboratories, Inc., Oberlin, Ohio) in a cell of 1-cm light path by using uninoculated gas equilibrated medium as reference. OD increases in short-term growth experiments were directly proportional to viable counts. The viable count calculated for an OD of 1 was 2.8×10^9 cells per ml in these cultures as well as in cultures grown to higher densities. Viable count, OD, and dry weight were proportional to an OD of 0.400. Cultures having higher densities were diluted to the proportional range, and the OD was calculated by using the dilution factor. The volume of the inoculum was adjusted as necessary to provide approximately the same initial cell concentration (5×10^7 cells per ml) in the fermentor in all experiments.

RESULTS

The composition of the gas atmosphere for growing *H. eutropha* autotrophically in shaken cultures had been chosen to provide the highest growth rate (6, 7). Under these conditions a 2- to 4-hr lag period was always observed prior to exponential growth when a 1% inoculum from postexponential phase or stationary phase cultures was used (Fig. 1). A slight lag period occurred when the inoculum was taken from an exponentially growing culture. The rate of exponential growth in all cultures was the same regardless of the type of inoculum used.

Reproducible growth responses described above were obtained with inocula from fresh cultures. Other experiments performed with stored inocula (stationary phase) were of incidental interest because they suggested effective methods of storing stock cultures. It was found that inocula changed within 3 days when stored at room temperature in flasks sealed with rubber stoppers, whereas inocula stored for a week in flasks with fitted cotton plugs grew like the fresh inoculum on subculture.

A simple experiment (Table 1) clearly illustrated that in some way aerobic conditions during storage were required to maintain cell viability and to prevent lysis. Samples of a culture were distributed as shown to provide different degrees of accessibility to air. When a large surface-to-volume ratio existed (lines 2 and 5), viable counts and OD did not change during 30 days storage. The viable count decreased 100-fold and lysis accounted for a 50% decrease in turbidity when a small surface-to-volume ratio existed. It was immaterial whether the tube was

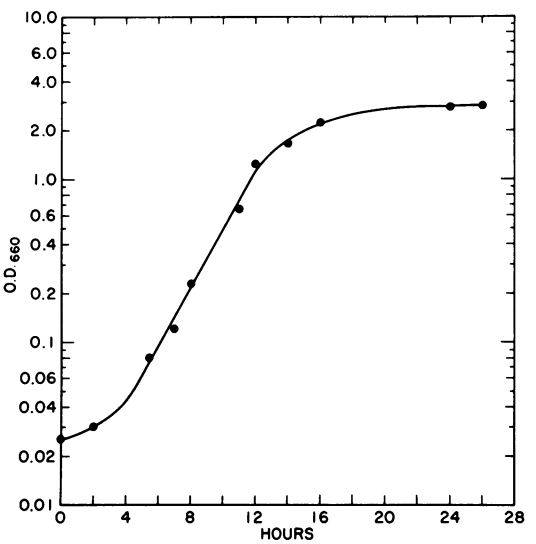

FIG. 1. *Growth curve of Hydrogenomonas eutropha cultured chemoautotrophically (100 ml) at 31 C on a gyratory shaker. The gas mixture contained 70% H_2, 20% O_2, and 10% CO_2.*

TABLE 1. *Effect of storage conditions on Hydrogenomonas eutropha*

Storage conditions[a]	*p*H	OD_{660}	Viable count/ml
None (original culture)	6.4	2.260	6.0×10^9
Flask (50-ml) cotton plug (10 ml)[b]	6.4	2.090	6.5×10^9
Test tube, screw cap (10 ml)	6.3	1.220	3.3×10^7
Test tube, plastic cap (10 ml)	6.4	1.170	6.7×10^7
Test tube, plastic cap (1.5 ml)	6.6	2.200	7.5×10^9

[a] 30 days at room temperature.
[b] Sample volume.

sealed (line 3) or had access to air (line 4). Even when cell viability was unchanged during storage, some subtle change occurred in the cells because on subculture the lag period extended to about 6 hr. Inocula used in the experiments described below were obtained from fresh cultures or from cultures stored aerobically for less than 1 week.

Effect of pO_2 and pCO_2 on initial growth. Larger volume cultures were grown in a 2.5-liter fermentor where adequate gas diffusion required sparged gases distributed by an impeller. Individual flow meters permitted accurate control and rapid modification of the gas mixture. Cultures grown in the standard medium with the usual gas mixture (70% H_2, 20% O_2, and 10% CO_2) had 18- to 24-hr lag periods. The lag periods became shorter as the partial pressures of O_2 and CO_2 were decreased to 0.05 atm, indicating excess oxygen, carbon dioxide, or both, were significant factors in the lag period.

The optimum pO_2 and pCO_2 for a minimum lag period were determined with three partial pressures of O_2 and CO_2 (0.025, 0.05, and 0.09 atm). In these experiments the gas mixture consisted of the indicated partial pressures of O_2 and CO_2; the remaining gas in the mixture was hydrogen. Although the pH_2 varied between experiments, it was in excess of 0.50 atm and was not limiting (1, 8). Because CO_2 altered the *p*H of the medium, the *p*H was adjusted during the equilibration period and was monitored during the experiment. During these short periods of growth, the adjusted *p*H remained constant.

Figures 2A, B, and C show that at *p*H 6.4 the inoculum grew without a lag period with most of the gas combinations tested, and the pO_2 and pCO_2 were much lower than was required for shake cultures. Most rapid exponential growth occurred with a pCO_2 of 0.05 atm and a pO_2 of either 0.05 or 0.09 atm. The optimum CO_2 concentration was easily defined, since the growth rates fell off sharply on either side of 0.05 atm. The optimum pO_2 range was not as narrow. A higher rate of growth was obtained with 0.09 atm of O_2 (μ = 0.33), but the longer sustained growth was obtained with 0.05 atm of O_2 (μ = 0.24; Fig. 2B), and this concentration of O_2 was considered optimal. With 0.05 atm of O_2 and CO_2, a break in the growth rate consistently occurred after about 3 hr; several hours thereafter, growth again resumed at the original rate. The break could be circumvented and the initial rate of growth could be maintained without interruption by adjusting the pCO_2 to 0.02 atm before the change in rate. Growth then continued at the same specific rate to OD of 10 if additional adjustments were made as the CO_2 and O_2 became limiting (*unpublished results*).

The growth rate was depressed and approximated that shown in Fig. 2B for 0.09 atm of CO_2 when the *p*H of the medium was increased only 0.31 unit to *p*H 6.71. In another series of experiments conducted at *p*H 6.71 with various partial pressures of CO_2, it was found that a pCO_2 of 0.025 atm (Fig. 2D) was needed to duplicate the former growth rate (μ = 0.24). When the *p*H of the medium was increased again by 0.32 units to *p*H 7.03, the pCO_2 required to achieve the same growth rate decreased again by half to 0.012 atm; both 0.05 and 0.025 atm CO_2 were inhibitory at this *p*H. In all of these experiments, the optimum pO_2 continued to be 0.05 atm.

For each incremental increase in *p*H of approximately 0.3 unit, it was found that a 50% decrease in pCO_2 was needed to obtain an equivalent growth rate; consequently, with an overall *p*H increase of only 0.6 unit, a fourfold reduction in pCO_2 was required. The *p*H change per se had no apparent effect on growth, since the same growth rates were achieved at each *p*H tested (Fig. 2D). These results are explained on the basis of the CO_2-bicarbonate ion equilibrium. At constant *p*H, the bicarbonate ion concentration is directly proportional to the pCO_2. With a constant pCO_2, the bicarbonate ion concentration in equilibrium with CO_2 doubles with each 0.3 *p*H unit increase (10). The empirically determined pCO_2 required to attain a given specific growth rate at the *p*H levels tested was the same as the calculated pCO_2 required to maintain a constant bicarbonate concentration of 1.6×10^{-3} M (10). Growth of the inoculum was therefore dependent upon a constant bicarbonate ion concentration and independent of the absolute pCO_2. Bicarbonate ion, not free CO_2, was critical in the first hours of growth of *H. eutropha*.

Effect of physiological age of the inoculum on the CO_2 requirement. Chesney (2) showed that the lag period observed on subculture was related to the physiological age of the inoculum. Cultures used as inocula in the previous experiments were harvested in the postexponential phase of growth (OD_{660} of 1.3 to 1.7; Fig. 1). Consequently, it was of interest to determine the effect of pO_2 and pCO_2 on initial growth with exponential phase cells (OD_{660} of 0.700) and with stationary phase cells (OD_{660} of 2.0 to 3.5). It is also known that the size of the inoculum influences the length of the lag period (5). This variable was controlled by adjusting the volume of the inoculum to provide the same final cell concentration throughout.

When exponential phase inocula were grown at *p*H 6.45 with 0.05 atm CO_2, the growth rate was very low, contrary to what was described above

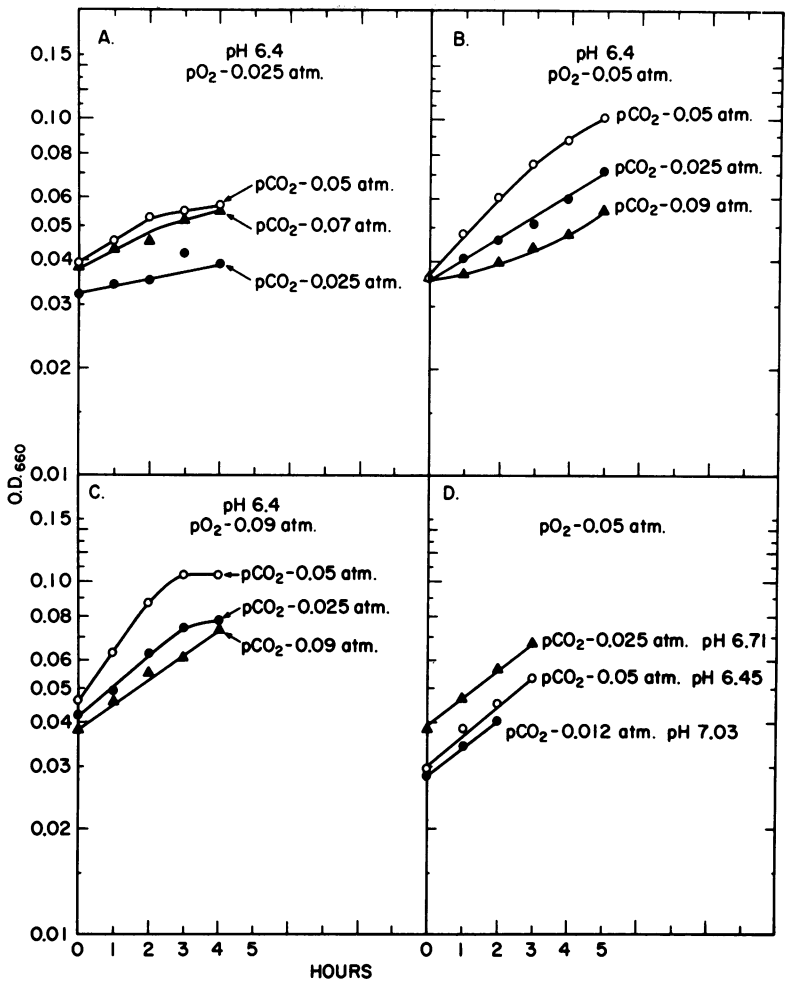

FIG. 2. *Initial growth response of Hydrogenomonas eutropha as a function of pO_2 and pCO_2 at 24 C. Gases were sparged through the cultures which were grown in a fermentor containing 1 liter of medium. The pH adjusted during gas equilibration remained stable during the experiment. A 2% postexponential inoculum having an OD_{660} of 1.6 was used.*

with postexponential phase inocula. As shown in Fig. 3, the desired rate of growth (μ = 0.24) was obtained when the pCO_2 was reduced 2.5-fold to 0.02 atm (6.6×10^{-4} M bicarbonate at equilibrium). Although the optimum pCO_2 was lower for exponential phase inocula, a similar adverse growth rate response occurred with half or twice the CO_2 concentration. The effect of increasing the *p*H to 6.71, while holding the CO_2 constant at 0.02 atm, is shown in Fig. 3. Growth was inhibited as a result of doubling the bicarbonate concentration. When the pCO_2 was reduced to 0.01 atm (*not shown*), growth occurred at the expected rate (μ = 0.24). It is equally valid to consider these results from the converse point of view; namely, at *p*H 6.71, 0.02 atm CO_2 provided excess bicarbonate, but by reducing the *p*H to 6.45 the bicarbonate concentration was decreased to the appropriate concentration.

Stationary phase inocula have the same pattern of growth responses to CO_2 as found with the other types of inocula. Quantitatively, stationary phase inocula required a much higher pCO_2 to begin immediate exponential growth at a specific growth rate of 0.24; 0.16 atm CO_2 (5.3×10^{-3} M bicarbonate at equilibrium) was optimal. The relationship between optimum pCO_2 and the *p*H of the medium with inocula from older cultures is shown in Fig. 4 at *p*H 6.40, 6.70, and 6.90. Note that since the *p*H increase from 6.70 to 6.90 was only 0.2 of a *p*H unit, the pCO_2 decrease required to maintain a bicarbonate concentration of 5.3×10^{-3} M was not 50% but approximately two-thirds of 50%. The

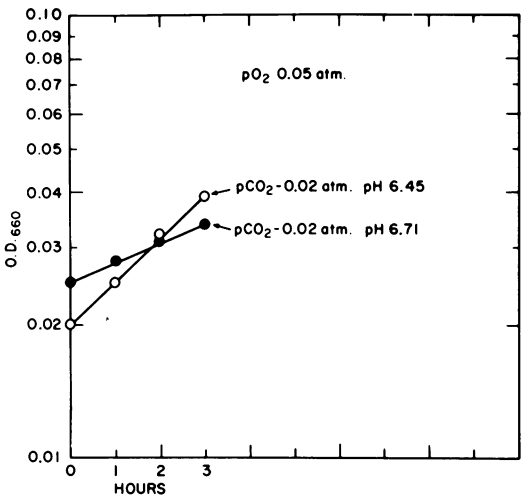

FIG. 3. *Same conditions as Fig. 2 except a 4% exponential phase inoculum having an OD_{660} of 0.72 was used.*

bottom curve in Fig. 4 illustrates the effect of suboptimal CO_2 (bicarbonate) on growth. A pCO_2 of 0.05 atm which was optimal for growth of stationary phase inocula at *p*H 6.90 was not effective at *p*H 6.40 because the *p*H change decreased the bicarbonate ion concentration to 1.6×10^{-3} M. At the time indicated by the arrow, the pCO_2 was raised to 0.16 atm resulting in an increase in the bicarbonate concentration to 5.3×10^{-3} M (*p*H simultaneously adjusted to maintain the *p*H at 6.40). Growth immediately responded by increasing to the expected rate.

DISCUSSION

A discrepancy existed between the O_2 and CO_2 concentrations required for the same initial growth rates of shaken cultures and of gas-sparged cultures. Considerably lower gas concentrations needed for sparged cultures indicated that gas medium equilibration was not achieved in shaken cultures even though a relatively small medium volume was used in a flask with baffles to increase effective surface area (7). These results raise a question about the validity of classifying hydrogenomonads on the basis of oxygen tolerance (*Bergey's Manual*, 7th ed.; 4) determined by various culture methods in different laboratories.

Equilibration of O_2 and CO_2 with the medium can only be assured by direct measure of dissolved gases. This kind of equipment was not available so experimental conditions were adopted which would effectively approach gas medium equilibration in sparged cultures. These included a 30-min gas medium pre-equilibration period, large gas flow rates, high impeller speeds, a relatively small inoculum, and a short experimental time limited to about two cell doublings. If the rate of gas uptake exceeded the rate of gas diffusion, (i) increased impeller speeds would have affected growth, (ii) the *p*H would have drifted due to CO_2 depletion as the cell concentration increased, or (iii) the pCO_2 requirement would have increased with time. The most sensitive indicator would have been CO_2 because a small change in the pCO_2 would have had a marked effect on the growth rate. None of these effects was observed. The change in rate of cell growth that did occur after several hours was not associated with a deficiency of one of the gases; it was caused by inhibition by excess CO_2. Inhibition was relieved by decreasing the pCO_2 to 0.02 atm (*p*H 6.4).

It was shown in Fig. 2B that the pCO_2 for a maximum rate of growth had narrow limits that were exceeded by plus or minus twofold changes in the CO_2 concentration. This fact permitted a clear establishment of the optimum CO_2 for growth of a postexponential phase inoculum at *p*H 6.40. The same response was found with exponential phase and with stationary phase inocula, but the absolute CO_2 requirement was different for each type of inoculum. At *p*H 6.40 to 6.45, exponential phase inocula required a pCO_2 of 0.02 atm (Fig. 3), postexponential phase inocula required 0.05 atm CO_2, and stationary phase inocula required 0.16 atm CO_2. The optimum pCO_2 for each inoculum was also related to the *p*H of the medium; for each 0.3 *p*H unit increase, a 50% reduction of the pCO_2 was re-

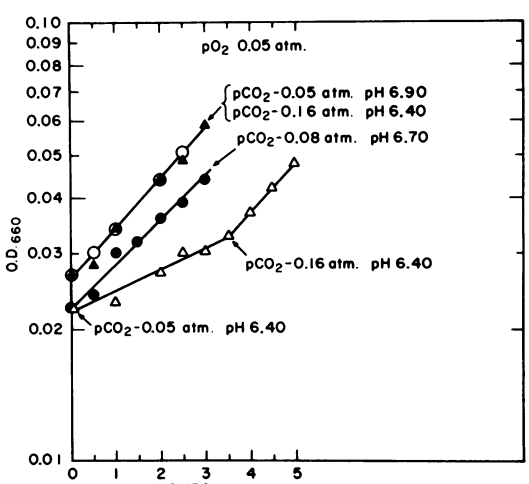

FIG. 4. *Same conditions as Fig. 2 except a 1.4% stationary phase inoculum having an OD_{660} of 2.330 was used. In the lowest curve, the pCO_2 was increased to 0.16 atm at 3.5 hr, and the pH was simultaneously adjusted to maintain 6.40.*

quired (Figs. 2D and 4). The inverse relationship of a decreasing CO_2 requirement with increasing *p*H of the medium showed that CO_2 was not the primary substrate. Calculation of the bicarbonate ion concentration in equilibrium with the optimum pCO_2 at each *p*H tested showed that growth was dependent upon a constant bicarbonate concentration. Immediate exponential growth of inocula from exponential, postexponential, and stationary phase cultures required 6.6×10^{-4} M, 1.6×10^{-3} M, and 5.3×10^{-3} M bicarbonate, respectively. As would be expected, the optimum bicarbonate ion concentrations could be established either by adjusting the pCO_2 at a given *p*H (Fig. 2 and 4) or, as shown in Fig. 3, by adjusting the *p*H (from *p*H 6.71 to 6.45) at a fixed pCO_2. The various bicarbonate requirements of inocula of different physiological age changed after the culture had grown for one or two generations, and all cultures then needed 6.6×10^{-4} M bicarbonate to maintain exponential growth. This was the same bicarbonate concentration needed initially by exponential phase inocula. Inocula from old cultures thus changed their metabolic requirements during the first hours of exponential growth.

These data show that, in some way, growth of inocula from older cultures can be initiated immediately at maximum specific rates by critical concentrations of bicarbonate. Subcultures of exponentially growing cells also required bicarbonate, implicating the same bicarbonate system. This was further confirmed by the similarity of the sharp optima shown for bicarbonate with all types of inocula tested. If the same bicarbonate system was involved in each case, it appears that this system becomes functionally degenerate during physiological aging. Progressively higher bicarbonate ion concentrations were therefore needed to compensate for the loss of activity. After one or two cell generations, the original activity was restored and a decrease in the bicarbonate concentration was required for continued growth.

Optimal growth conditions of *H. eutropha* (and other cells) generally are those which provide the highest growth rate and the maximum extent of growth (6, 7). The assumption made is that cells in the inoculum and cells in exponentially growing cultures have the same qualitative and quantitative nutritional requirements. It has been shown that this assumption is applicable only to the special case where the inoculum is from an exponentially growing culture. Since older inocula of *H. eutropha* have a quantitatively different requirement for bicarbonate than is provided in the usual growth medium, a lag period is inevitable.

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