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Control of *Escherichia coli* Growth by CO_2

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Escherichia coli B dependence on CO_2 for growth was demonstrated. At suboptimal CO_2 concentrations the rate of growth was controlled by CO_2 concentration.

Many techniques used to minimize the lag period preceding culture growth were described in the early microbiological literature, whereas others are a part of the legacy of unpublished observations from many laboratories. The appearance of a lag period after successive transfers of cultures in the same medium always was an enigma that has been subject to a number of theories, but each hypothesis was unable to account for some experimental observations. While determining conditions to obtain reproducible growth of the obligate autotrophic aerobe, *Alcaligenes eutrophus*, we found that a specific concentration of one component, bicarbonate, produced immediate growth without the customary lag period (6). This prompted a comparative study with an organism at the opposite end of the nutritional scale. Anaerobically grown *Streptococcus sanguis* responded in the same way, but CO_2 was the active species (7). In this report we show that growth of a washed stationary-phase inoculum of the facultative aerobe, *Escherichia coli*, also will grow without a lag period if a suitable concentration of CO_2 is provided. With all three organisms the initial and subsequent exponential growth rate was controlled solely by the concentration of CO_2 or bicarbonate. These findings indicate that the effect of CO_2 or bicarbonate on the conventional lag period and on the growth rate is not an unusual phenomenon and suggest that CO_2 or bicarbonate may have the same regulatory function in all microorganisms.

Carbon dioxide or its hydrated form, bicarbonate, has been a recognized requirement for cell growth since Valley and Rettger (8) performed an extensive survey and demonstrated that none of the more than 100 microorganisms tested would grow if CO_2 were excluded. More recently the basis for the CO_2 requirement by heterotrophic microorganisms was established; CO_2-fixing enzymes were isolated and their functions in various metabolic pathways are now known. The qualitative CO_2 requirement for growth is not the subject of this report. We wish to emphasize the fundamental physiological effect of CO_2 concentration on regulation of the growth rate. The lag period appears to be the initial manifestation of the same effect.

Walker (9) first showed that added CO_2 shortened the lag period of *E. coli*, and Neidhardt et al. (5) recently reported that the lag period was virtually eliminated when bicarbonate was incorporated in the medium. Quantitative demonstration of the effect of CO_2 on growth requires a minimal medium and a system for accurately maintaining low concentrations of CO_2. Small incremental increases in CO_2 concentration must be tested to assure detection of the optimum, for the optimum may span a narrow range of concentrations (6, 7).

A vessel with a 1-liter working capacity was designed to receive three separate gases (N_2, O_2, and CO_2) which were mixed in the vessel to form an atmosphere of any desired composition (6, 7). Gas flow rates were individually regulated by calibrated flow meters. A maximum flow rate of 10 liters/min and vigorous impeller action decreased equilibration time and minimized retention of metabolic CO_2. The incubation temperature was 37°C. *E. coli* B (ATTC 11303), used in these experiments, was grown aerobically in glucose-minimal medium (1). The inocula for the test cultures were grown at 37°C in a water bath shaker for 8 to 9 h at which time they were in stationary phase for 2 to 3 h. The cultures were then stored at 4°C overnight.

An aliquot of the culture to be used as inoculum was washed twice in 0.001 M phosphate buffer (pH 6.0), resuspended in the same buffer, bubbled with N_2 to remove CO_2, and added to the culture vessel containing medium equilibrated with the starting gas mixture. Growth was measured continuously by optical density at 660 nm (OD_{660}), by cell count and size distribution (Coulter Counter), and by total protein (4).

The CO_2 requirement for growth is shown in Fig. 1. A stored and washed stationary-phase inoculum, which would exhibit a lag in ordinary shaken cultures, immediately grew when CO_2

was included in the gas atmosphere (Fig. 1, curve A); however, the same inoculum did not grow without added CO_2 when metabolic CO_2 was not permitted to accumulate (Fig. 1, curve B). The lag ended abruptly with addition of CO_2, and a constant exponential growth rate was rapidly established. This rate was maintained only as long as CO_2 was provided; growth ceased again when CO_2 was eliminated from the gas mixture.

In the experiments illustrated in Fig. 1, a saturating concentration of CO_2 was used. The rate of growth was a function of CO_2 concentration between 0.0025% (the lowest concentration tested) and 0.03 to 0.04% CO_2 where saturation occurred. Direct control of the rate of growth can be exercised by control of the CO_2 concentration as shown in Fig. 2. The unambiguous responses to changes in the low concentrations of CO_2 and the facility with which a given growth rate can be selected and maintained in batch culture is evident.

Growth rates determined with limiting CO_2 concentrations at different pH values established that *E. coli* responded to CO_2 concentration, not to bicarbonate ion concentration (6, 7).

The data in Fig. 3 validate the use of optical density as a measure of culture growth under these experimental conditions. It also illustrates the effect of CO_2 on protein synthesis. Changes in net protein paralleled changes in OD that were influenced by the presence or absence of CO_2. Total protein and OD increased in tandem whereas cell counts initially remained essentially unchanged; later, when CO_2 addition was discontinued, OD and protein increases stopped whereas the cell count continued to increase for a time. Cell size distribution analyses reflected the consequences anticipated under these circumstances. Small cells in the stationary-phase inoculum increased in size before cell division occurred (2, 3). A constant cell size was then maintained as cell numbers increased exponentially, but after CO_2 was removed, the average cell size decreased again as cells continued to divide in the absence of net protein synthesis.

E. coli requires CO_2 for growth. When a saturating concentration was present at the time of inoculation, growth proceeded at the maximum rate (Fig. 1 and 3). Limiting concentrations of CO_2 restrict the rate of growth in proportion to the concentration of CO_2 present (Fig. 2). It follows that a freshly inoculated aerobic medium equilibrated with atmospheric CO_2 (approximately 0.02%) would be limiting in CO_2 and would support a submaximum growth rate. As metabolic CO_2 increased the effective CO_2 concentration in the medium, a period of increasing growth rates, i.e., a lag period, would be observed. There are obvious factors which would

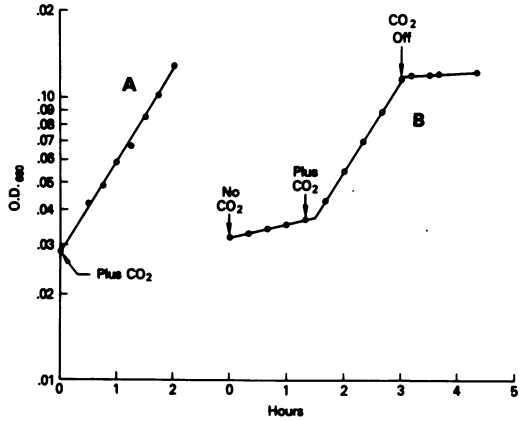

FIG. 1. *E. coli B growth requirement for CO_2. Stored and washed stationary-phase cells were inoculated into a glucose-minimal medium (1) equilibrated with a gas atmosphere created by adjusting the relative flow rates (to 10 liters/min of total flow) of individual cylinder gases (N_2, O_2, and CO_2) to provide the proportions desired. O_2 was maintained at 4%. Curve A, A 0.075% CO_2 final concentration was present throughout. Curve B, No CO_2 was present at zero time, but 0.3% CO_2 (final concentration) was added where indicated and was deleted again after the interval shown. The CO_2 concentrations used in both experiments were not limiting for growth. Doubling times were 55 min. Incubation temperature was 37°C; pH 6.4.*

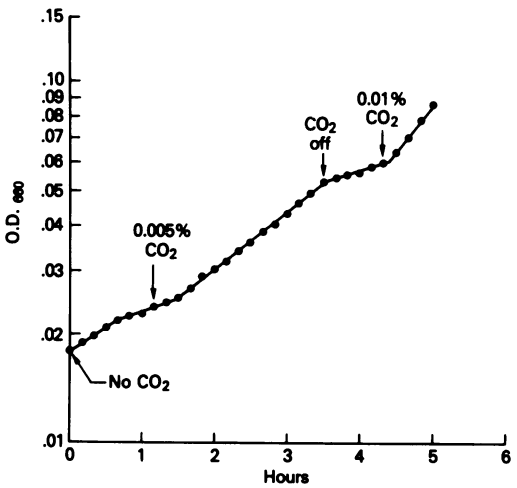

FIG. 2. *Regulation of E. coli B growth rates in minimal medium by control of CO_2 concentration. Washed stationary-phase cells were inoculated into glucose-minimal medium equilibrated with 4% O_2 and no CO_2. Control of the gas atmosphere was as in Fig. 1. At times indicated, CO_2 was added to the other flowing gases to produce the final concentration shown or it was deleted (CO_2 off). Doubling times were: 245 min before 0.005% CO_2 was added, and 115 min after addition; 295 min after CO_2 was deleted, and 75 min in the presence of 0.01% CO_2. Incubation temperature was 37°C; pH 6.4.*

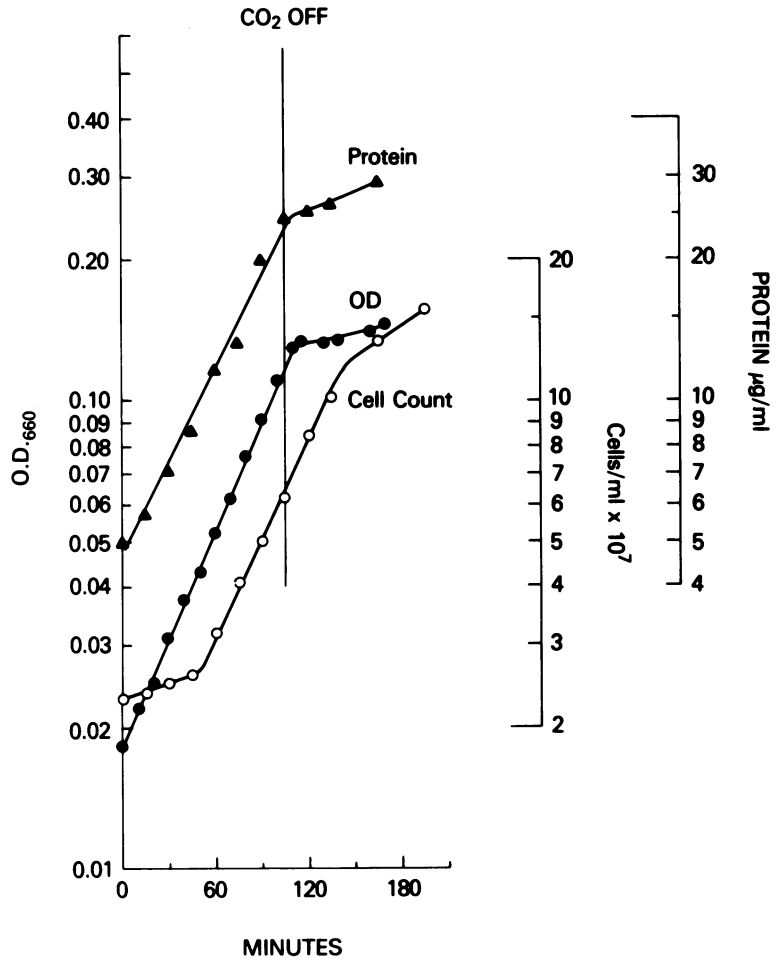

FIG. 3. *Growth responses of a stationary-phase E. coli B inoculum to CO_2. Treatment of stationary-phase inoculum and culture conditions as described in Fig. 1. Initial CO_2 concentration was 0.3%; the O_2 concentration was 4%. Samples for cell count and size distribution (Coulter Counter) and net protein were chilled immediately and analyzed. Incubation temperature was 37°C; pH 6.4.*

accelerate or delay establishing a saturating CO_2 concentration. A large inoculum, a metabolically active inoculum, and a "rich" medium would favor a short lag period, whereas excessive aeration by shaking or sparging would tend to dissipate metabolic CO_2 and extend the lag period. These effects have been observed by most microbiologists, but the cause has not been attributed to the effect of CO_2 concentration on control of growth.

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