Carbon Dioxide Control of Lag Period and Growth of *Streptococcus sanguis*

ROY REPASKE, ANNE C. REPASKE, AND RON D. MAYER

Laboratory of Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014, and Department of Biology, Catholic University, Washington, D.C. 20017

Received for publication 30 July 1973

A carbon dioxide requirement for growth of *Streptococcus sanguis* was readily demonstrated in a fermentor where the gas atmosphere could be controlled. Growth at a maximum rate occurred immediately in response to the appropriate CO₂ concentration; growth stopped when CO₂ was deleted. Washed inocula consisting of exponentially growing cells required a minimum of 2.4% CO₂, postexponential phase cells needed 1.2 to 1.8% CO₂ immediately and 2.4% CO₂ shortly thereafter, whereas stationary phase cells required three sequential increases in CO₂ from 0.3 to 1.8 to 2.4% within the first 90 min of growth. These CO₂ concentrations permitted each inoculum to initiate growth immediately at the same maximum rate. These results also showed that physiologically "old" cells had the same capacity for growth as "young" cells when the CO₂ concentrations were appropriate for the type of inoculum. Continued exponential growth of the culture at the same optimum rate required 2.4% CO₂. Lower concentrations of CO₂ were rate limiting and the resulting exponential rate was proportional to the CO₂ concentration. The "normal" lag period of *S. sanguis* appears to be an artifact induced by a CO₂ deficiency.

A carbon dioxide requirement for *Streptococcus* and *Lactobacillus* growth has been noted repeatedly (3, 7, 13, 15, 16, 19, 20, 21, 28) since Valley and Rettger first reported the effect in 1927 (25). The general need for CO₂ has been demonstrated among streptococci representing many groups (7, 15, 20, 28). However, the CO₂ requirement may be obscured when media containing casein hydrolysate are used (21) since some species of *Streptococcus* derive CO₂ indirectly by the action of arginine dihydrolase on arginine (7, 8, 21, 27). With these organisms, growth continued only until arginine was exhausted, but such limitations on growth did not occur when ornithine plus CO₂ was substituted for arginine (7, 23). Interchangeable growth-promoting effects of CO₂, biotin, and oleic acid have been reported for certain species of *Streptococcus* and *Lactobacillus* (3, 5, 6, 13, 18). In the above organisms as well as in *Escherichia coli*, C¹⁴O₂ tracer studies were used to determine the fate of incorporated CO₂. Carbon dioxide was fixed exclusively or predominantly in the β carboxyl of aspartic acid (1, 10, 13, 16-18, 28) suggesting that CO₂ fixation was needed for the synthesis of aspartic acid.

The carbon dioxide requirement has not been restricted to cell growth; it has been shown to be

required for proteinase biosynthesis by non-proliferating cells of *Streptococcus faecalis* var. *liquificans* (23, 27) and by cell-free extracts (11). Welch (27) showed that exogenously supplied aspartic acid could replace CO₂ in non-proliferating cells. Demonstration of pyruvic carboxylase and transaminase activities in cell extracts of *S. faecalis* established the presence of enzymes which could account for CO₂ fixation in the synthesis of aspartic acid (11).

These observations fit the concept for the need for CO₂ fixation by heterotrophs to replenish C₄ dicarboxylic acids for anaplerotic requirements of cells (12).

In this investigation we show that *S. sanguis* was dependent upon CO₂ for growth. The rate of growth could be predictably controlled at limiting CO₂ concentrations. The manifestation of a CO₂ deficiency at the time of inoculation was a lag period. Stationary phase inocula grew immediately without a lag period when appropriate CO₂ concentrations were provided.

MATERIALS AND METHODS

S. sanguis (group H strain Challis, originally from R. Pakula, University of Toronto through R. M. Cole, National Institutes of Health) was grown in MS-6, a complex defined medium containing glucose as the

energy source (14); the medium was modified by the addition of aspartic acid (200 mg per liter). The medium was sterilized by filtration. Bicarbonate (tube cultures only) and cysteine were added prior to inoculation. For most of the reported studies, inocula were stationary phase cells grown in screw-capped test tubes at 37 C for 8 h (initial pH 8.2, final pH, 5.4) (Fig. 1). Cultures were stored overnight at 4 C before use.

Growth experiments were performed anaerobically in a specially designed fermentor having a working volume of 1 liter (22). Purified cylinder gases were added separately to the fermentor vessel through spargers located under the impeller. The flow rate of each gas was independently regulated by a calibrated flow meter (Brooks Instrument Div.; Emerson Electric Co., Hatfield, Pa.) and the percentage composition of the gas atmosphere was calculated from the flow rates. The constancy of the gas atmosphere was assured by maintaining continuous high gas flow rates (5 liters total flow per min). In experiments reported here, prepurified nitrogen was constantly passed through the medium during periods of no CO₂ addition to prevent accumulation of any endogenously formed CO₂ or to sweep out CO₂ previously added. The pH of the medium was monitored and adjustments were made as necessary in response to CO₂ changes.

Demonstration of a CO₂ requirement necessitated the removal of traces of dissolved CO₂ and bicarbonate from the medium. A liter of medium in the assembled fermentor was acidified to pH 4.5 with H₂SO₄, and CO₂ was purged by flushing N₂ through the medium and head space for 30 min. Cysteine (200 mg per liter) and Antifoam AF (Dow Corning Corp.,

Midland, Mich.) were added, flow of the desired gas(es) was established, and the pH was readjusted to the starting pH with carbonate-free NaOH. After 20 to 30 min for gas equilibration, the medium was inoculated. Even with precautions to eliminate contamination by CO₂, a slow rate of growth (doubling time 6 to 9 h) was sometimes observed in a "CO₂ free" medium. In other experiments no growth occurred. It is believed that the low rate of growth without CO₂ resulted from technical difficulties in removing traces of CO₂ and/or from endogenous CO₂. The effects of added CO₂ on growth were so pronounced that a slow base line rate with no CO₂ did not complicate interpretations.

The inoculum consisted of a sufficient quantity of stationary phase cells to give an initial optical density (OD₅₅₀) of 0.020 to 0.025. The inoculum was washed in sterile 0.01 M phosphate buffer, pH 7, just before inoculation. The incubation temperature was 37 C. Culture purity was confirmed after culture growth.

In early experiments evaluation of growth during the first 2 h was difficult because culture samples were prone to clump when mixed in air, causing irregular optical density readings that prevented establishing a slope. This property of cells was not confined to *S. sanguis*; young cultures of *Hydrogenomonas eutropha* and *E. coli* behaved similarly. Clumping was eliminated and stable optical density values were obtained (Fig. 2 to 7) by modifying the fermentor for continuous monitoring of the optical density in a closed system. Occasionally, an irregularity in optical density was observed immediately after inoculation (Fig. 3C). Optical densities were determined on small portions of the culture which were constantly circulated by a peristaltic pump to a Gilford Flow-Through (Gilford Instrument Labs., Inc., Oberlin, Ohio) spectrophotometer cell and back to the fermentor. A de-bubbling chamber was interposed before the cell. Optical densities were measured and recorded by a Gilford spectrophotometer (model 2400) which automatically compensated for uninoculated medium as reference.

Variations in generation times between repeated experiments with different inocula were usually ± 5 min, but occasionally they were as high as ± 10 min. Growth in the fermentor was carried to OD 1.5 in some experiments. Independent plate counts established that viable colony-forming units and optical density were proportional. An optical density of 1.0 corresponded to 9.5×10^8 colony-forming units per ml.

RESULTS

S. sanguis is a nutritionally fastidious organism. Excellent growth (Fig. 1) occurred in tube or stationary flask cultures in a defined complex medium (14) which contained 18 amino acids, adenine, guanine, uracil, a variety of vitamins, and salts. Little or no growth resulted when cultures were grown in a fermentor through which prepurified nitrogen was sparged (5 liters per min). This dramatic inability of the orga-

FIG. 1. Growth curve of *S. sanguis* grown at 37 C in tube culture containing bicarbonate.

FIG. 2. Effect of CO_2 concentration on initiation of growth of *S. sanguis* in fermentor cultures at 37 C and pH 7.15. Washed stationary phase cells were used to inoculate media (1 liter) which had been pre-equilibrated for 30 min with CO_2 at the concentration indicated. The remainder of the gas atmosphere was N_2 . (g = generation time.)

FIG. 3. Requirement by stationary phase inocula for sequential increases in CO_2 concentrations to maintain a constant growth rate during the early hours of growth. The medium in the fermentor was pre-equilibrated with 0.3% CO_2 before inoculation. Subsequent changes in CO_2 concentration were made at the times indicated. Other conditions as in Fig. 2.

nism to grow in the fermentor in the same medium was shown to be due to the absence of carbon dioxide. When both CO_2 and N_2 were sparged through the medium, growth rates

comparable to those shown in Fig. 1 were achieved.

It was found that initial CO_2 requirements of cultures inoculated with stationary or pre-stationary phase cells were different from the CO_2 requirements after several hours of growth. Demonstration of these CO_2 effects required a fermentor which had the capability of rapid adjustment and accurate control of the CO_2 concentration.

FIG. 4. Growth of stationary phase inocula at pH 6.25 and the effect of deleting and readmitting CO_2 . Other conditions as in Fig. 3.

FIG. 5. Response of *S. sanguis* to different CO_2 concentrations after preincubating cells for 30 min in medium without CO_2 . Changes in CO_2 concentrations are indicated by arrows. Other conditions as in Fig. 3.

FIG. 6. Response of *S. sanguis* to different CO₂ concentrations after preincubating cells for 60 to 90 min in medium without CO₂. Conditions as in Fig. 5.

Effect of CO₂ after inoculation. The mandatory requirement for CO₂ for initiation of rapid growth is illustrated in Fig. 2. Little or no growth occurred without added CO₂ in the first 2 h as shown in Fig. 2A; in other experiments CO₂ was withheld for 5 h with the same results.

The responses of stationary phase inocula of *S. sanguis* to various CO₂ concentrations during the first hours of incubation are shown in Fig. 2B to E. Initial growth rates increased as the CO₂ increased to 0.3%; thereafter, the initial rates progressively decreased with increased CO₂ concentrations. Two-phased curves were always observed with 0.3, 1.2, and 2.4% CO₂. Initial rates decreased after 1 to 1.5 h with 0.3 and 1.2% CO₂, whereas the growth rate increased with 2.4% CO₂ and became equal to the initial rate with 0.3% CO₂. These results indicated that the CO₂ requirement had changed (with growth or with time) from 0.3 to 2.4% and suggested that a constant growth rate could be maintained by starting with 0.3% and changing to 2.4% CO₂. When this was attempted, an intermediate discontinuity in growth rate invariably occurred. However, a three-step increase

in the CO₂ concentration from 0.3 to 1.8 to 2.4% accomplished the desired results (Fig. 3C and 4). Figure 3B shows that the intermediate CO₂ concentration (1.8%) could not sustain the higher rate. No further changes in CO₂ concentration (after 2.4%) were required for continued growth of the culture. When exponential phase inocula were used, optimum growth rates were achieved with 2.4% CO₂ at the time of inoculation. Inocula derived from cultures intermediate between exponential and stationary phases required 1.2 to 1.8% CO₂ followed by 2.4% CO₂ if maximum rates of growth were to be maintained. These data show a progressive change in CO₂ requirement with the physiological age of the inoculum. In all instances, CO₂ controlled the lag period and the appropriate concentrations of CO₂ eliminated the lag period. The CO₂ requirement then remained 2.4% for each of the above cultures, and early differences among different types of inocula no longer were observed.

CO₂ requirement to maintain extended growth. Growth was not sensitive to higher concentrations of CO₂ after the CO₂ requirement became established at 2.4%. The CO₂ concentration could be increased to as much as 15% (pH maintained by base addition) without inhibition. With 20% CO₂, spontaneous bubble formation in the cuvette prevented reliable readings. At the other end of the scale, the growth rate remained exponential but de-

FIG. 7. CO₂ effect on growth of stationary phase inocula preincubated 1 h under nitrogen in 0.01 M phosphate, pH 6.9, at the indicated temperatures. After centrifugation, cells were resuspended and added to medium pre-equilibrated with 2.4% CO₂. Other conditions as in Fig. 2.

creased proportionally as the CO_2 was decreased below 2.4%. Growth at a desired exponential rate was possible by selecting a limiting CO_2 concentration. Growth rates were not affected by pH changes within the pH range tested: pH 6.25 (Fig. 4) to pH 8.05.

S. sanguis continued to require CO_2 throughout growth. When CO_2 addition to the culture was discontinued, growth essentially stopped (Fig. 3B and 4 for pH 7.15 and 6.25, respectively) and growth resumed immediately when CO_2 was again supplied (Fig. 4). The delay before growth stopped depended upon the time required to sweep out CO_2 from the head space of the fermentor and upon the pH of the medium. The higher the pH of the medium the higher the concentration of bicarbonate in the medium in equilibrium with the CO_2 . This reservoir of CO_2 at pH 7.15 with 1.8% CO_2 was 3×10^{-3} M bicarbonate, sufficient bicarbonate to supply CO_2 for growth for 50 additional min (Fig. 3B). At pH 6.25 with 2.4% CO_2 , the bicarbonate concentration was 4.9×10^{-4} M or sixfold less, and growth persisted only for 15 min (Fig. 4).

CO_2 versus bicarbonate requirement. It is possible to distinguish between a CO_2 and a bicarbonate requirement if growth rates are altered by moderate changes in CO_2 concentrations. At a fixed pH, the bicarbonate ion concentration is directly proportional to the CO_2 concentration (at equilibrium). The bicarbonate ion concentration can be selectively changed while the CO_2 concentration is held constant by changing only the pH of the medium, or conversely, the bicarbonate concentration can be held constant by varying the CO_2 concentration according to the pH of the medium (24). When these variations were applied to autotrophically growing *H. eutropha*, it was found that growth of this organism responded to bicarbonate concentration, not to CO_2 concentration (22).

At pH 7.15 (Fig. 2) distinct differences in growth rates of *S. sanguis* were observed with 0.15 and 0.3% CO_2 . This represented a twofold difference in CO_2 as well as bicarbonate ion concentrations. The same experiments with the same CO_2 concentrations were repeated at pH 6.25; consequently, only the bicarbonate ion concentrations were altered and were eightfold less (24). Rates and pattern of growth at pH 6.25 were the same as for pH 7.15, showing that *S. sanguis*, unlike *H. eutropha*, responded to the CO_2 concentration. Experiments conducted at pH 8.05 gave identical results.

Evaluation of the initial CO_2 requirement. There was no immediate explanation for stationary phase inocula needing successive in-

creases in CO_2 concentrations. Although the need changed during growth there was no evidence that it was contingent upon growth. The question could be answered by direct test in the presence of medium because growth did not occur in the absence of CO_2 . Stationary phase inocula were prepared in the usual way. Washed cells were first incubated for 30 min in medium under N_2 at 37 C after which CO_2 was introduced at a concentration of 0.3, 1.8, or 2.4% (Fig. 5). The CO_2 requirement had clearly changed during the 30-min incubation without CO_2 . Addition of 0.3% CO_2 now initiated growth at a lower rate than obtained when 0.3% CO_2 was present at the time of inoculation (Fig. 2C). The curves indicate that the optimum CO_2 had increased to somewhat less than 1.8%. Similar experiments in which CO_2 addition was delayed for 60 to 90 min revealed that the optimum CO_2 concentration had now increased to 2.4% (Fig. 6). Therefore, during a 60- to 90-min incubation period without CO_2 , the optimum CO_2 concentration progressively changed from 0.3% to 2.4% in the same way as with growing cells even though no significant cell growth occurred.

Was the change in CO_2 requirement influenced by the medium, by the loss of some diffusible substance from the cells, or by changes in the cells resulting from endogenous metabolism? Sufficient stationary phase inocula for two cultures were washed three times in cold 0.01 M PO_4 buffer, pH 7, to remove easily diffusible substances. The final 10-ml suspension of cells in buffer was divided into two portions. One sample was placed in an ice bath, the other in a 37 C water bath. Both were bubbled with CO_2 -free nitrogen for 60 min before inoculating the media pre-equilibrated with 2.4% CO_2 (Fig. 7).

The inoculum preincubated in ice had changed slightly but had the same general characteristics as in Fig. 2E where no pretreatment had occurred. The inoculum preincubated at 37 C had lost the requirement for graded increases in CO_2 and began growing rapidly in 2.4% CO_2 . Inocula treated in the same way were added to media equilibrated with 0.3% CO_2 ; again the ice preincubated cells were unchanged since they grew rapidly in 0.3% CO_2 (as in Fig. 2C), whereas the 37 C preincubated cells grew at a reduced rate in 0.3% CO_2 . These results showed that neither nutrients from the medium nor loss of some easily diffusible substance washed from the cells was responsible for the required changes in CO_2 concentrations. During incubation at 37 C in phosphate buffer, cells either metabolized or released a substance(s) which resulted in a progressively increasing

demand for higher concentrations of CO₂. When present, this substance also caused inhibition with elevated CO₂ concentrations (2.4%), a condition that no longer prevailed after 2.4% CO₂ was optimum (15% CO₂ was not inhibitory).

Two compounds, oleate and aspartate, have been reported to spare or replace the CO₂ requirement for some streptococci and lactobacilli (3, 5, 6, 13, 18). In media supplemented with oleate (0.4 mg/liter), the CO₂ requirements for *S. sanguis* were not eliminated or modified at any time during growth. Growth interrupted by deleting and resupplying CO₂ was unchanged. It has been assumed that CO₂ fixation by heterotrophs was required primarily to produce a C₄ dicarboxylic acid (2, 12) which became a precursor of aspartic acid (1, 10, 12, 13, 16, 17, 18, 28). Cells permeable to aspartate would need CO₂, or aspartate, but not both (2, 27). Since the medium in these studies with *S. sanguis* contained aspartate, comparisons were made in this medium minus aspartate. Stationary phase inocula were prepared as described. Growth rates after the first hour were identical with and without aspartate and cultures grew to the same extent. A considerable difference was observed during the first hour. Whereas the 0.3, 1.8, 2.4% CO₂ sequence prevented the lag period when aspartate was present, the lag period persisted when aspartate was absent. Various CO₂ concentrations were tested between 0.3% and 12%; the initial rate tended to increase but never attained the maximum rate. These data indirectly indicate that aspartate entered the cell and its effectiveness was restricted to the first hour of growth. Aspartate does not replace CO₂; it must be present to permit CO₂ to function in eliminating the lag period.

DISCUSSION

The response of growth to relatively small changes in CO₂ concentration has not been reported until recently (4, 22) apparently because CO₂ was not thought of as a usual substrate, much less one whose optimum concentration might be confined to narrow limits. The known general requirement for CO₂ for growth of microorganisms (25) has caused little concern about supplying CO₂ as a nutrient; most organisms have a net production of metabolic CO₂ which ultimately can satisfy the requirement. For that very reason, demonstrating a CO₂ requirement is often difficult.

In a defined medium considered to be nutritionally complete (14), total growth of *S. sanguis* in 8 h was essentially the same with or without bicarbonate in the medium (Fig. 1). Under more rigorously controlled conditions in

a fermentor, where adventitious CO₂ could be removed from the medium before inoculation and metabolic CO₂ could be swept out rapidly after inoculation, *S. sanguis* could not grow at any substantial rate unless CO₂ was supplied. Fermentor cultures required 2.4% CO₂ to sustain long-term growth at the same maximum rate of growth that was observed in test tube cultures with added bicarbonate (as in Fig. 1). Since *S. sanguis* required CO₂ for growth, sufficient metabolic CO₂ must have been produced in the tube culture to provide a minimum of 2.4% CO₂. The organism's capacity to produce CO₂ would explain why it was difficult to demonstrate zero growth rates under N₂ in the fermentor. Generation times without CO₂ (6 h and longer) were very long compared to the 40- to 50-min generation times with an optimum CO₂ concentration. This fact and the observed responses of the culture to added CO₂, to variations in CO₂ concentrations and to deletion of CO₂ are the basis for the interpretation that growth of *S. sanguis* was dependent upon CO₂.

Detection of these CO₂ effects required a culture apparatus in which (i) the medium could be freed of CO₂ before inoculation, (ii) metabolically produced CO₂ could be flushed out by high flow rates of inert gas (N₂), (iii) flow rates of each gas could be independently and accurately controlled, and (iv) vigorous agitation could rapidly reestablish gas-liquid equilibrium when the gas atmosphere was changed. Evidence that these conditions were met was shown by the essential absence of growth before CO₂ was added (Fig. 2, 5, and 6) and the prompt growth and the stability of the growth rate after CO₂ was added (Fig. 4, 5, and 6). The less rapid termination of growth after discontinuing CO₂ addition (Fig. 3B and 4) was due to limitations of the system. Residual bicarbonate in the medium (established by the CO₂-bicarbonate equilibrium) provided a gradually decreasing reservoir of CO₂ as discussed above (Results). The length of time growth continued after CO₂ addition was stopped would be expected to correspond to the amount of bicarbonate present in the medium.

The CO₂ requirement for growth of *S. sanguis* immediately after inoculation should be considered separately from the subsequent requirement. During the first 60 to 90 min after inoculation, the CO₂ requirement may progressively change depending upon the physiological age of the inoculum. Exponential phase inocula grew immediately at maximum rates with 2.4% CO₂. Cultures inoculated with cells past the exponential phase but not yet in stationary phase also grew at the same rate if 1.2 to 1.8%

CO₂ was provided initially, followed by an increase to 2.4% CO₂. Stationary phase inocula required a three-step increase from 0.3 to 1.8 to 2.4% CO₂ (Fig. 2-4) if the maximum rate of growth was to be maintained. After the CO₂ requirement became 2.4%, all cultures responded similarly and the original growth rate was maintained to an OD₆₀₀ exceeding 1.0 without further changes in CO₂ concentration.

Within the first hour of growth with stationary phase inocula, a shift in CO₂ from 0.3 to 2.4% inhibited the growth rate (Fig. 2C, E and Fig. 5B, C). In contrast, when the optimum CO₂ concentration had become established at 2.4%, any increase in CO₂ up to 15% had no effect on the growth rate. Lesser concentrations of CO₂, however, were growth rate limiting. Growth remained exponential and decreased proportionally as the CO₂ concentration decreased below 2.4%. Growth stopped when CO₂ addition was discontinued (Fig. 3B and 4) and began again promptly when CO₂ was added (Fig. 4).

Growth restricted by limiting CO₂ can be seen in Fig. 6. These cells after 60 min of incubation at 37 C without CO₂ had a demonstrable requirement for 2.4% CO₂ (Fig. 6D) and gave responses analogous to cultures that had gone through the 0.3, 1.8 to 2.4% CO₂ sequence (Fig. 4). Culture growth rates can be controlled as shown by controlling the CO₂ concentration.

Growth of a routine laboratory culture of *S. sanguis* (Fig. 1 and ref. 14) invariably was preceded by a lag period which could be empirically shortened by using a large inoculum or an exponential phase inoculum, or both. Stationary phase cells are examples of cells most likely to have long lag periods. In the experiments reported here we have shown that a relatively small inoculum of washed stationary phase *S. sanguis* will grow without a lag period at the maximum exponential rate if the appropriate CO₂ concentrations are provided.

Carbon dioxide control of the lag period and of subsequent growth was not confined to *Streptococcus*. *E. coli* grown aerobically in glucose minimal medium required CO₂ for growth (2). We found the lag period disappeared when the appropriate CO₂ concentration was supplied (unpublished results). The importance of CO₂ in relation to the lag period is emphasized by the results obtained with another organism at the opposite extreme of the nutritional scale, *H. eutropha*. Autotrophically grown *H. eutropha* did not respond to CO₂ but to the equivalent hydrated species, bicarbonate (22). With proper bicarbonate concentrations, inocula of any physiological age also began growing at maximum rate without a lag period. Walker (26)

proposed a relationship between the lag period and CO₂ concentration, but his data still showed lag periods several hours in duration.

From results of the three organisms studied it was shown that physiologically old cells have the same capacity for immediate rapid growth as physiologically young cells. This capacity was controlled only by the initial CO₂ (bicarbonate) concentration. Usual culture conditions do not include an external supply of CO₂, so microorganisms that require CO₂ must provide the prerequisite CO₂ metabolically in the new medium. A lag period is assured until the CO₂ concentration has reached the saturating level for the particular organism.

Under growth conditions where control of the CO₂ concentration was possible, oleate did not substitute for CO₂ or spare the CO₂ requirement of *S. sanguis*. Aspartate was reported to completely relieve the CO₂ requirement of "minute" streptococci (18) but not of *Streptococcus bovis* (28). *S. bovis* preferentially used aspartic acid produced intracellularly via CO₂ fixation even though exogenous aspartate was present. Permeability characteristics could explain this difference since *S. bovis* was able to grow in a simple medium, whereas most other streptococci have extensive nutritional requirements and presumably broad permeability capabilities. Aspartate did not replace or reduce the 2.4% CO₂ required for extended growth of *S. sanguis*, nor did it affect the rate of growth. Aspartate did affect growth during the first 60 to 90 min when it was required together with CO₂ to remove the lag period. These results as well as those with *H. eutropha* suggest that physiologically old cells have some alteration of a system responsive to CO₂.

ACKNOWLEDGMENTS

We wish to express our appreciation to David I. Brand for making plate counts and to Daniel N. Arshack for technical assistance.

LITERATURE CITED

1. Abelson, P. H., E. T. Bolton, and E. Aldous. 1952. Utilization of carbon dioxide in the synthesis of proteins by *Escherichia coli* I. *J. Biol. Chem.* **198**:165-172.
2. Aji, S. J., and C. H. Werkman. 1948. Replacement of CO₂ in heterotrophic metabolism. *Arch. Biochem.* **19**:483-492.
3. Broquist, H. P., and E. E. Snell. 1951. Biotin and bacterial growth. I. Relation to aspartate, oleate and carbon dioxide. *J. Biol. Chem.* **188**:431-444.
4. Dehority, B. A. 1971. Carbon dioxide requirement of various species of rumen bacteria. *J. Bacteriol.* **105**:70-76.
5. Deibel, R. H., and C. F. Niven, Jr. 1955. Reciprocal replacement of oleic acid and CO₂ in the nutrition of the "minute" streptococci and *Lactobacillus leickmannii*. *J. Bacteriol.* **70**:134-140.
6. Deibel, R. H., and C. F. Niven, Jr. 1955. The minute

- streptococci: further studies on their nutritional requirements and growth characteristics on blood agar. *J. Bacteriol.* **70**:141-146.
7. Gale, E. F. 1945. The arginine, ornithine and carbon dioxide requirements of *Streptococci* (Lancefield Group D) and their relationship to arginine dihydrolase activity. *Brit. J. Exp. Pathol.* **26**:225-233.
 8. Gale, E. F. 1945. The effect of sulfanilamide on the arginine and carbon dioxide requirements of *Streptococci* (Lancefield Group D). *Brit. J. Exp. Pathol.* **26**:234-247.
 9. Gladstone, G. P., P. Fildes, and G. M. Richardson. 1935. Carbon dioxide as an essential factor in the growth of bacteria. *Brit. J. Exp. Pathol.* **16**:335-348.
 10. Goff, R. C., and R. E. Hartman. 1970. Carbon dioxide fixation by cells of *Streptococcus faecalis* var *liquefaciens*. *J. Bacteriol.* **104**:27-33.
 11. Hartman, R. E. 1970. Carbon dioxide fixation by extracts of *Streptococcus faecalis* var *liquefaciens*. *J. Bacteriol.* **102**:341-346.
 12. Kornberg, H. L. 1966. The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochem. J.* **99**:1-11.
 13. Lardy, H. A., R. L. Potter, and R. H. Burris. 1949. Metabolic functions of biotin. I. The role of biotin in bicarbonate utilization by *Lactobacillus arabinosus* studied with C¹⁴. *J. Biol. Chem.* **179**:721-731.
 14. Leonard, C. G., J. M. Rånhand, and R. M. Cole. 1970. Competence factor production in chemically defined media by non-competent cells of Group H *Streptococcus* strain Challis. *J. Bacteriol.* **104**:674-683.
 15. Liu, P. 1954. Carbon dioxide requirement for group F and minute colony G hemolytic streptococci. *J. Bacteriol.* **68**:282-288.
 16. Lynch, V. H., and M. Calvin. 1952. Carbon dioxide fixation by microorganisms. *J. Bacteriol.* **63**:525-531.
 17. MacDonald, J. C. 1958. Synthesis of aspartic acid by *Lactobacillus arabinosus*. *Can. J. Microbiol.* **4**:335-343.
 18. Martin, W. R., and C. F. Niven, Jr. 1960. Mode of CO₂ fixation by the minute streptococci. *J. Bacteriol.* **79**:295-298.
 19. Niven, C. F., Jr., M. R. Washburn, and J. M. Sherman. 1946. Folic acid requirements of the "minute" Streptococci. *J. Bacteriol.* **51**:128-129.
 20. Platt, T. B., and E. M. Foster. 1958. Products of glucose metabolism by hemofermentative streptococci under anaerobic conditions. *J. Bacteriol.* **75**:453-459.
 21. Prescott, J. M., and A. L. Stutts. 1955. Effects of carbon dioxide on the growth and amino acid requirements of *Streptococcus bovis*. *J. Bacteriol.* **70**:285-288.
 22. Repaske, R., C. A. Ambrose, A. C. Repaske, and M. DeLacy. 1971. Bicarbonate requirement for elimination of the lag period of *Hydrogenomonas eutropha*. *J. Bacteriol.* **107**:712-717.
 23. Swiencicki, J. F., and R. E. Hartman. 1967. The effects of oxygen and carbon dioxide on proteinase biosynthesis by *Streptococcus faecalis* var *liquefaciens*. *Can. J. Microbiol.* **13**:1445-1450.
 24. Umbreit, W. W., R. W. Burris, and J. F. Stauffer. 1957. Manometric techniques. 3rd. ed. Burgess Publishing Co., Minneapolis.
 25. Valley, G., and L. F. Rettger. 1927. The influence of carbon dioxide on bacteria. *J. Bacteriol.* **14**:101-137.
 26. Walker, H. H. 1932. Carbon dioxide as a factor affecting lag in bacterial growth. *Science* **76**:602-604.
 27. Welch, P. C., and R. E. Hartman. 1969. Effect of carbon dioxide on the aspartic acid requirement for proteinase biosynthesis by *Streptococcus faecalis* var *liquefaciens*. *J. Bacteriol.* **100**:1138-1139.
 28. Wright, D. E. 1960. The metabolism of carbon dioxide by *Streptococcus bovis*. *J. Gen. Microbiol.* **22**:713-725.