# Carbon Dioxide Effects on Glucose Catabolism by Mixed Microbial Cultures[1]

PETER E. GAFFNEY

*Georgia Institute of Technology, Engineering Experiment Station, Atlanta, Georgia*

## ABSTRACT

GAFFNEY, PETER E. (Georgia Institute of Technology, Atlanta). Carbon dioxide effects on glucose catabolism by mixed microbial cultures. Appl. Microbiol. **13**:507–510. 1965.—Results have shown that, with mixed culture (sewage) inocula, the lag period in aerobic catabolism of glucose can be reduced by increased $CO_2$ tension. Conversely, removal of $CO_2$ from the air supply to the growth flasks and Warburg vessels may increase the lag period.

It has long been known that $CO_2$ is necessary for the nutrition of autotrophic bacteria (Bonazzi, 1921; Gowda, 1924). That many heterotrophic organisms are capable of fixing $CO_2$ is generally known, but possible benefit of fixation is little understood.

Valley and Rettger (1926) presented a list of 82 organisms for which $CO_2$ appeared to be necessary for development. In many instances, they found that growth could be prevented by removing the $CO_2$ from the atmosphere with which the organism was supplied. After studying more than 100 different organisms representing various families and genera, they concluded that $CO_2$ is necessary for the growth and development of the bacterial cell (Valley and Rettger, 1927).

Walker (1932) confirmed the work of Valley and Rettger and demonstrated that $CO_2$ had its greatest effect during the lag phase. He suggested that the phenomenon of lag was due to the time needed for the culture to build up necessary $CO_2$ content. This focused attention on the possibility of a definite $CO_2$ requirement for heterotrophs. In an attempt to explain this requirement, Werkman and Wood (1942) postulated a condensation of pyruvate and $CO_2$ to form oxaloacetic acid, indicating its critical importance with respect to the citric acid cycle. Krampitz and Werkman (1941) showed some support for this, and more definite evidence on an enzymatic basis was later shown by Kaltenbach and Kalnitsky (1951).

During a previous investigation (Gaffney and Heukelekian, 1961) dealing with comparison of oxidation rates of the lower fatty acids under various conditions in a Warburg respirometer,

[1] A portion of this paper was presented to the Southeastern Branch of the American Society for Microbiology, Jacksonville, Fla., 18 October 1963.

long lag periods were encountered, and it was shown that these could not be eliminated or significantly reduced by altering the quality ("adapting") or the quantity of inocula.

It was suggested (Pardee, 1949; Krebs, 1951) that the results obtained with the Warburg technique are often in error due to the elimination of $CO_2$ with the direct method.

The objective of this investigation was to determine the effect of the presence or absence of $CO_2$ on the initiation and rate of catabolism in a dispersed aeration system, and also the effect on oxygen-uptake rates, as measured by use of the Warburg respirometer.

## MATERIALS AND METHODS

The medium consisted of basal salts, phosphate buffer ($pH$ 7.0), and 1,000 mg per liter of glucose, and was inoculated (1.0% by volume) with various settled domestic sewage samples. The mixture was divided into three equal samples, each receiving different air supplies. Fritted-glass bubblers were inserted into the system between the main air supply and the growth flasks (Fig. 1). The bubblers contained water, a supersaturated solution of sodium bicarbonate, or 20% potassium hydroxide, so that the air delivered to the growth flasks was normal, $CO_2$-supplemented, or $CO_2$-free, respectively. $CO_2$-free air was demonstrated by precipitation tests with barium hydroxide. In three of the tests, a $CO_2$ gas tank instead of the bicarbonate solution was used for supplementation.

Glucose catabolism was measured daily by analysis of chemical oxygen demand (COD) according to the 11th edition of *Standard Methods for Analysis of Water and Wastewater* (American Public Health Association, 1960). The COD analysis of a substrate consists of complete chemical combustion of organic substances by refluxing in acid dichromate, and the value is expressed as the

maximal amount of oxygen consumed in destruction of the organic material.

In the respirometric experiments, Dickens-Simer flasks were used with the routine potassium hydroxide $CO_2$ trap and compared with similar systems containing diethanolamine as a $CO_2$ buffer, as suggested by Krebs (1951). Use of the diethanolamine under these conditions results in constant maintenance of a 1 to 2% $CO_2$ atmosphere in the Warburg vessels.

## RESULTS

The results with five different sewage inocula are shown in Table 1. The COD values are averages of triplicate analyses, and the experimental error is ±4.0%. During test IV, after aeration for 1 day, total bacterial plate counts (on glucose-agar) were made on the solutions from the three growth flasks. The buffer held the pH in each test between 6.9 and 7.2. Only the results after 1 day
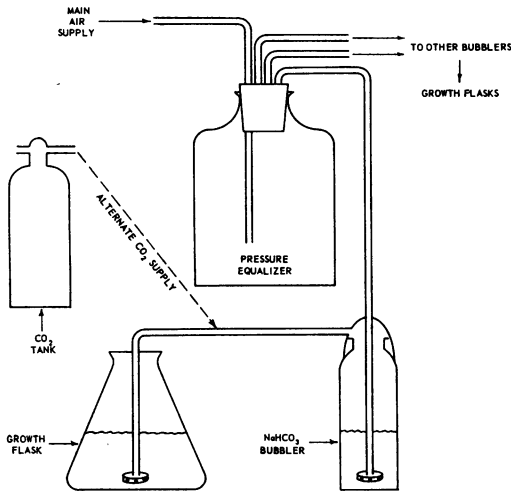
of aeration are given, because the early period is of most interest.

The data from the first four tests consistently show that a greater percentage of glucose COD was dissimilated after 1 day when $CO_2$ was added or left in the air supply than when $CO_2$-free air was supplied. Also, the growth activity (in terms of numbers of organisms) followed the same pattern. The percentage of glucose COD dissimilation for water, $CO_2$, and KOH was 5.2, 33, and 0.9%, respectively; the corresponding bacterial counts were 34,000,000, 80,000,000, and 15,000,000 organisms per milliliter. The COD re-

TABLE 2. *Increased glucose COD dissimilated with normal and $CO_2$-enriched air as compared with $CO_2$-free air*

| Test no. | Normal air | $CO_2$-enriched air |
|----------|-----------|---------------------|
| I | +180* | +280 |
| II | +50 | +670 |
| III | +10 | +110 |
| IV | +50 | +370 |
| V | −13 | −12 |
| Mean increase | +55 | +284 |

* Results expressed as milligrams per liter.



FIG. 1. *Gas supply system.*

TABLE 1. *Amount of glucose COD dissimilated after aeration for 1 day**

| Test no. | Water Amt† | Water Per cent | Bubbler NaHCO₃ Amt | Bubbler NaHCO₃ Per cent | $CO_2$ gas Amt | $CO_2$ gas Per cent | KOH Amt | KOH Per cent |
|------|------|------|------|------|------|------|------|------|
| I | 390 | 34.0 | 490 | 43 | | | 210 | 18 |
| II | 50 | 4.4 | 670 | 58 | | | 0 | 0 |
| III | 40 | 3.5 | | | 140 | 12 | 30 | 2.6 |
| IV | 60 | 5.2 | | | 380 | 33 | 10 | 0.9 |
| V | 12 | 1.0 | | | 13 | 2 | 25 | 3.0 |

* Initial glucose, 1,000 mg per liter; initial COD, 1,150 mg per liter.

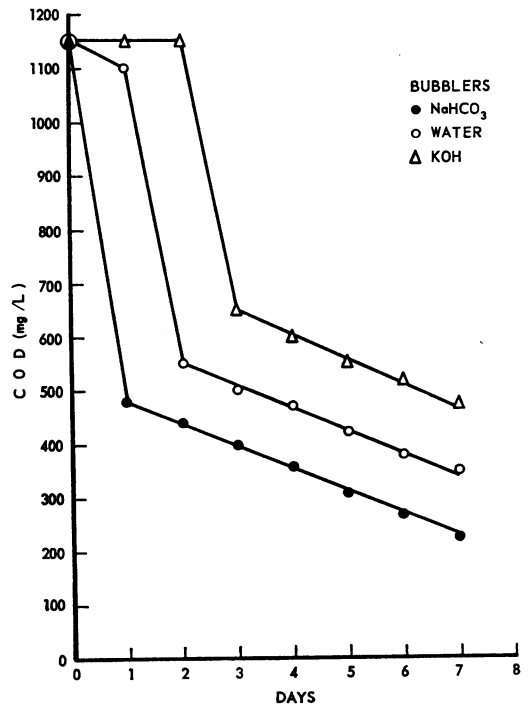† All amounts are expressed as milligrams per liter.



FIG. 2. *Dissimilation of 1,000 mg per liter of glucose.*

TABLE 3. *Effect of CO₂ on oxygen uptake of various mixtures in a Warburg respirometer*

| Substrate mixture | Oxygen uptake in 20 hr | |
|---|---|---|
| | No CO₂* | With CO₂† |
| | *μliters* | *μliters* |
| 100% domestic sewage....... | 100 | 210 |
| 1% sewage + 50 mg per liter of glucose................... | 44 | 80 |
| 1% sewage + 500 mg per liter of glucose................. | 0 | 100 |

\* Routine procedure with KOH trap.
† Diethanolamine "buffer" was used (1 to 2% CO₂ atmosphere).

sults of test V do not follow that pattern, and, in this test, CO₂ had no effect on the 1-day values. The data in Table 2 are derived from those in Table 1. Here it is shown that, compared with CO₂-free air, normal air allowed for an additional 55 mg per liter of glucose to be dissimilated in the first day, and CO₂-enriched air resulted in an increase of 284 mg per liter on the average.

The complete 7-day curves during test II are presented in Fig. 2. The bulk of catabolic activity occurred during the first day with CO₂-supplemented air, during the second day with normal air, and not until the third day with CO₂-free air.

Oxygen uptake was measured in the Warburg flasks for 20 hr with several substrate mixtures; the data in Table 3 are the result of measurement on three replicates of the mixture with and without CO₂. In each case, the oxygen uptake in the first 20 hr is much greater in the presence of CO₂ than in its absence, especially when the ratio of substrate to cells is high.

## DISCUSSION

In the past few years, there have been numerous reports of the beneficial nature of CO₂ to the heterotrophic bacteria. Harris (1954) found, by using constant concentrations of CO₂ (0.8%), that the oxygen uptake by dilute suspensions of 38 common bacterial species utilizing glucose as substrate was greater than oxygen uptake in flasks in which the CO₂ had been removed by concentrated alkali. In dilute suspensions, the stimulation seemed to be inversely related to the amount of CO₂ produced by the cells; when many cells were present, this stimulation was not observed.

Farghaly (1950) reported that carbon dioxide was an essential factor for the growth of luminous bacteria. Holm (1954) found that *Actinobacillus actinomycetemcomitans*, which does not normally grow on media under aerobic or anaerobic conditions, would grow on the surface of some solid

media under both conditions if a concentration (0.5% or more) of CO₂ was present.

Lui (1954) reported that the growth of hemolytic streptococci can be markedly improved by increased CO₂ concentrations. Maximal growth was achieved by most strains when the concentrations reached 5% and could not be improved by higher concentrations.

Mattman (1954), in comparing the growth of 869 cultures on blood-agar plates, showed that a significant percentage of strains grew only when CO₂ was added. The cultures included especially β-hemolytic streptococci, pneumococci, and micrococci.

Szulmajster (1958) reported that an organism belonging to the genus *Clostridium*, which was isolated from sewage sludge, was able to grow on creatinine (which is found in large percentages in sewage) only in the presence of CO₂ and yeast extract. It was also shown with labeled CO₂ that the CO₂ was incorporated into the cell material.

Field and Lichstein (1958) reported that autoclaved glucose medium produced an unidentified factor which satisfied the CO₂ requirements of propionibacteria for early initiation of growth.

In the early development of the standard-dilution, 5-day biochemical oxygen demand (BOD) test for the strength of sewage, Theriault (1931) reported somewhat higher results when phosphate buffer dilution water was used in place of a suggested bicarbonate water containing 300 mg per liter of sodium bicarbonate. Although this may have been indicative that CO₂ had no particular effect on the lag period, it is also likely that the sewage samples contained enough CO₂ to fill the requirement. Furthermore, the differences in Theriault's results could have been due to pH variations, as he stated that "it does not appear advisable to recommend the use of readily prepared bicarbonate solution for dilution purposes until more data from different laboratories have been accumulated regarding the effect of pH on the rate of deoxygenation in natural waters."

It was shown (Gaffney, *unpublished data*) that, in standard-dilution BOD tests on glucose and sewage, CO₂ supplementation of the phosphate buffer dilution water presently used had no effect (of reducing lag periods) on short-term BOD values (1 to 5 days). It was concluded that, with the relatively low concentrations of organic material used (10 mg per liter), the CO₂ requirement was not critical, since pronounced lag periods were absent. Thus, it is likely that the differences in the lag-reducing effects of CO₂ between dilution BOD tests and the investigations reported herein can be explained by the relative food-to-organisms ratios.

CO₂ supplementation of phosphate dilution

water in standard-dilution BOD tests may merit some investigation in cases of relatively resistant industrial wastes in which long lag periods may be observed. Also, since the Warburg respirometer is frequently used to obtain BOD values on numerous types of concentrated wastes, and since a low concentration of organisms is used, it would seem that the use of the common KOH absorbent in the reaction vessels would affect the validity of the data, particularly if the results are evaluated in terms of natural conditions in a stream.

The experiments described in this report were performed with dilute suspensions and sewage inocula containing a large variety of microorganisms. The beneficial effect of $CO_2$ supplementation has been demonstrated on the basis of the ability of mixed cultures to catabolize the soluble organic nutrient, specifically to reduce the COD at a greater initial rate. Corollary data indicated that the early phase of oxygen uptake in the Warburg flasks is similarly enhanced in the presence of increased $CO_2$ tension. On the basis of this and previous work, it would appear that $CO_2$ supplementation is important with respect to lag periods in catabolic measurements with heterotrophic organisms.

### ACKNOWLEDGMENTS

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