EFFECT OF CO_2 ON THE GROWTH RATE OF THE PNEUMOCOCCUS

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The role of carbon dioxide as an essential factor in bacterial growth, has been studied repeatedly since Wherry and Ervin (1918) found that the tubercle bacillus does not grow on agar when the CO_2 above the medium is absorbed by sodium hydroxide. The gonococcus (Chapin 1918), the meningococcus (Cohen and Fleming 1918, Kohmann 1919) and *Brucella abortus* (Huddleson 1921, T. Smith 1924, 1926, McAlpine and Slanetz 1928) were found to grow better in a higher CO_2 concentration than that of air. Rockwell and his associates (1921, 1923, 1924, 1926, 1927) confirmed these findings in a large number of aerobic and anaerobic organisms, including a strictly anaerobic pneumococcus, cultured from a suppurative knee joint on sugar-free ascites agar. Growth occurred in a nitrogen/carbon dioxide mixture, but failed to occur in a stream of nitrogen without CO_2, and in nitrogen in the presence of 10 per cent sodium hydroxide. Valley and Rettger (1926, 1927) compared the number of colonies on agar plates of over 100 strains of microorganisms in atmospheric and in CO_2-free air, and found in all instances a complete absence of growth in the presence of a carbon-dioxide-absorbent. Among the bacteria in their table, Valley and Rettger list a culture of *Encapsulatus pneumoniae* grown, with $Ca(OH)_2$ present, on sugar-free ("coli fermented") agar of pH 6.8.

Valley (1928) has reviewed comprehensively all the work up to 1928, carried out mostly on solid media and without maintaining very definite and constant gas concentrations. One of the first workers on this subject (Chapin 1918), for instance, writes: "A lighted candle left in the jar beside the plates at the time of sealing the chamber, I have found a convenient means of establishing a highly favorable atmosphere." Walker (1932) used liquid

media containing lactose, ammonium phosphate, and ammonium tartrate for culturing *Escherichia coli*. In a current of CO_2-free air, no growth occurred in 24 hours; when aeration was stopped, normal growth occurred in a few hours. The lag period in bacterial cultures was explained by Walker as the time the bacteria need to produce the necessary CO_2 concentration by their own metabolism.

Winslow, Walker and Sutermeister (1932) showed that growth was not prevented in *E. coli* by the absence of CO_2 when broth containing peptone or lactose was used as culture medium. Gladstone, Fildes and Richardson (1935) examined the effect of CO_2 on the growth of *E. coli*, two strains of *Eberthella typhosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus anthracis*, *Clostridium sporogenes*, *Clostridium welchii*, *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa*, using liquid culture media saturated with CO_2-free air, atmospheric air, and 10 vol. per cent CO_2/air, and with CO_2-free nitrogen and 10 vol. per cent CO_2/nitrogen respectively. They found that the presence of CO_2 was a prerequisite for the growth of all the bacteria examined, unless the culture media contained peptone. Davies (1940) examined the effect of various CO_2 concentrations on the growth of tubercle bacilli, strain H 37, in a phosphate-bicarbonate medium containing glycerol and asparagine. He found the growth optimum at 2.5 vol. per cent CO_2.

The growth-promoting effect of carbon dioxide on the numerous bacteria cited, indicates that also for heterotrophic (non-photosynthetic) systems, carbon dioxide is not merely an indifferent end-product of cell metabolism, but an essential metabolite or substrate. Direct evidence of this is given by various findings: Formic acid can be synthesized from CO_2 and H_2 by *E. coli* (Woods 1936); CO_2 is reduced to methane in connection with the fermentation of aliphatic alcohols (Barker 1936); propionic acid bacteria synthesize succinic acid from glycerol and carbon dioxide (Wood and Werkman 1936, 1938, 1940, Phelps, Johnson, and Peterson 1939); the formation of succinic acid during the fermentation of pyruvate, glucose, and galactose by *E. coli* is dependent upon the CO_2 partial pressure (Elsden 1938). Ruben, Kamen,

Carson, Barker, and Beck (1940) studied the carbon dioxide reduction of yeast, *E. coli*, barley root, rat liver, propionic acid bacteria, methane-producing bacteria, and *Clostridium acidi-urici* by the use of radioactive carbon, and showed that all of these "have the ability to convert carbon dioxide into organic constituents of their cells". Gaffron wrote 1937: "A chemical reduction of carbon dioxide is possible in principle in any living cell". (See also: Franck and Gaffron 1941.)

This paper deals with the effect of CO_2 on the growth rate of the pneumococcus.

EXPERIMENTAL

The experiments were carried out with the Warburg technique. Pneumococcus types I, II, and III were examined. Beef infusion, 0.5 per cent glucose, 1 per cent Difco-bacto-peptone broth, was used as culture medium, without any buffer, as well as with either 0.01 to 0.03 M phosphate or 0.01 to 0.025 M bicarbonate added. In some experiments citrated sheep blood was added to the broth (1 ml. blood to 9 ml. broth). Conical or rectangular manometer vessels of 18 to 20 ml. capacity contained the cultures (2 to 6 ml.) in the main space. For the experiments in a CO_2-free atmosphere, the side bulbs, or the insert wells of the vessels contained 0.2 to 0.5 ml. 20 per cent NaOH. The pneumococcus is very sensitive to changes of O_2 concentration (Schlayer 1936), and so it is necessary in the aerobic experiments to keep the O_2 tension constant throughout. The gas mixtures containing various CO_2 concentrations in a 20 vol. per cent O_2/nitrogen mixture for the aerobic, or in nitrogen without oxygen for the anaerobic experiments, were prepared over mercury (2.5 liters) and the culture vessels saturated with these mixtures while shaken in the thermostat at 38°C. The rate of growth was determined by measuring the aerobic oxygen consumption or anaerobic CO_2 production of the cultures by the "one vessel" or by the "two vessel" method. For determining rough differences in the number of bacteria, the photronreflectometer (Libby) was used.

Figure 1 shows the results of a manometric determination of the

growth rate of pneumococcus type I at pH 7.8 and 7.4, illustrating the well known fact that the growth rate of the pneumococcus is increased, at a pH between 7 and 8, in the more alkaline milieu (Dernby and Avery 1918, Cullen and Chesney 1918, Avery and Cullen 1919, Fennel and Fisher 1919). As a culture medium, standard 0.5 per cent glucose, 1 per cent peptone, beef infusion broth, without any buffer added, was used. The initial density

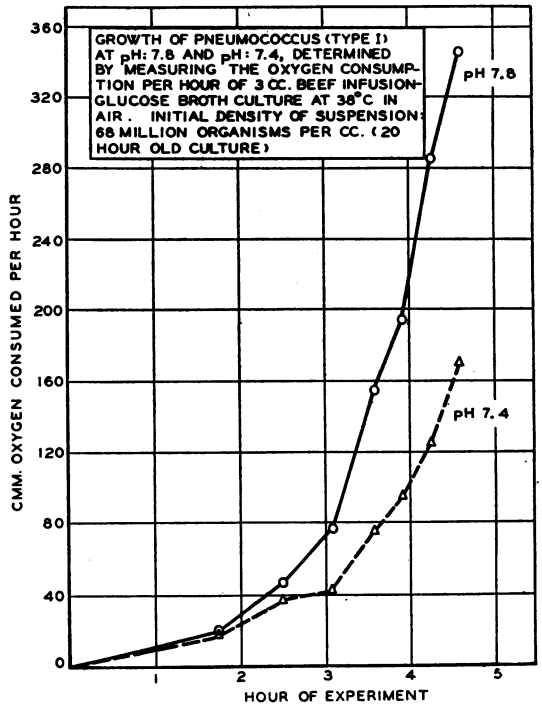

FIG. 1

of the bacteria broth was 68 million organisms per ml. The CO_2 concentration in both vessels was that of air. After 4½ hours the oxygen consumption per hour was at pH 7.8 345 cmm., at pH 7.4 170 cmm., indicating an increase of 100 per cent in the pneumococcus growth rate at the more alkaline pH. This basic fact, that the growth rate of pneumococci at pH 7.4 is decreased as compared with that at pH 7.8, must be borne in mind in interpreting all

the experiments, where, through higher CO_2 concentrations in an otherwise constant medium, the pH is shifted to the more acid side. If the CO_2 concentrations as such had no influence on the growth rate of pneumococci, figure 1 should lead us to anticipate that the shift to the acid side, caused by higher CO_2 concentrations, would result in a decrease of the growth rate; but actually the reverse is true.

Figure 2 shows a typical experiment with a pneumococcus type I culture in 0.03 M phosphate (original pH: 7.8), 0.5 per cent glu-

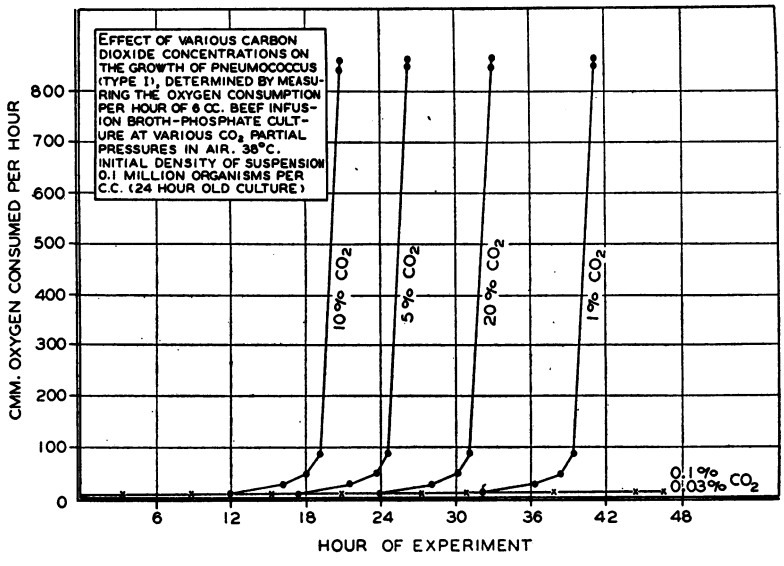

FIG. 2

cose, 1 per cent peptone, beef infusion broth at CO_2 concentrations of 0, 0.03, 0.1, 1, 5, 10, and 20 vol. per cent. The initial density of the suspension was about 0.1 million organisms per ml. The culture was 24 hours old. The initial CO_2 formation of the 6 ml. culture in the lag period was about 0.056 cmm. per hour, so that in the course of 24 hours the CO_2 formation of the 6 ml. suspension in the vessels with a gas space of about 13000 cmm. was about 1.3 cmm. Hence, in 24 hours this change in the original CO_2 concentration amounts to less than 0.02 vol. per cent. The initial pH of the suspension was 7.8 and remained 7.8 in the CO_2-

free culture. In the cultures with higher CO_2 concentrations, the pH was correspondingly more acid, so that with regard to the pH, the conditions were increasingly less favorable for pneumococcus growth. However, it is apparent from the curve that the growth-promoting effect of CO_2 is so much greater than the inhibiting effect of the more acid pH, that up to a concentration of 10 vol. per cent CO_2, in spite of the unfavorable pH, the pneumococcus cultures begin to grow earlier than at a lower CO_2 concentration and a pH closer to their pH optimum. The 10 vol. per cent culture reaches the logarithmic phase of multiplication after 21 hours; the 5 vol. per cent CO_2 culture after 26 hours; the 1 vol. per cent culture after 41 hours. That, even at a CO_2 concentration as high as 20 vol. per cent, with the corresponding considerable lowering of the pH, growth still occurs earlier than in the 1 vol. per cent CO_2 culture, though later than in the 5 vol. per cent and 10 vol. per cent CO_2 cultures, proves that an essential role is played by CO_2 in the growth rate of the pneumococcus. As the experiment shows, at a CO_2 concentration of 0 to 0.1 vol. per cent no growth occurred at all.

This prevention of growth in complete absence of CO_2 can only be overcome by using relatively large inocula (above 3 million organisms per ml.) and preferably in a culture medium improved by the addition of blood or serum, but under such conditions also, the growth-stimulating effect of higher CO_2 concentrations can be demonstrated. Figure 3 shows the growth rate of pneumococcus type I in blood, 0.2% glucose, 1% peptone, beef infusion broth, determined by measuring the anaerobic CO_2 formation at various CO_2 concentrations in nitrogen. The initial density of the suspension was 6 million organisms per ml. The culture was only 2 hours old. The bicarbonate concentration was 0.01 M in all vessels. The CO_2 concentration was 1.5, 2.5, and 4 vol. per cent, the pH respectively being 7.59, 7.37, 7.17. In this anaerobic experiment too, the more acid medium as such would have caused a decrease in the growth rate, but it is obvious from the curve that the growth-stimulating effect of the higher CO_2 concentrations greatly exceeds the growth-inhibiting effect of the more acid pH, so that in the final result, the competition of the two growth-in-

fluencing factors leads to a marked predominance of the CO_2 effect. After a little over 4 hours, the CO_2 formation per hour of the 2 ml. culture is at 4 vol. per cent CO_2 323 cmm., at 2.5 vol. per cent CO_2 274 cmm., at 1.5 vol. per cent CO_2 173 cmm. It is thus seen that there is an increase of 87 per cent in the number of bacteria at 4 vol. per cent CO_2 compared to that at 1.5 vol. per cent CO_2.

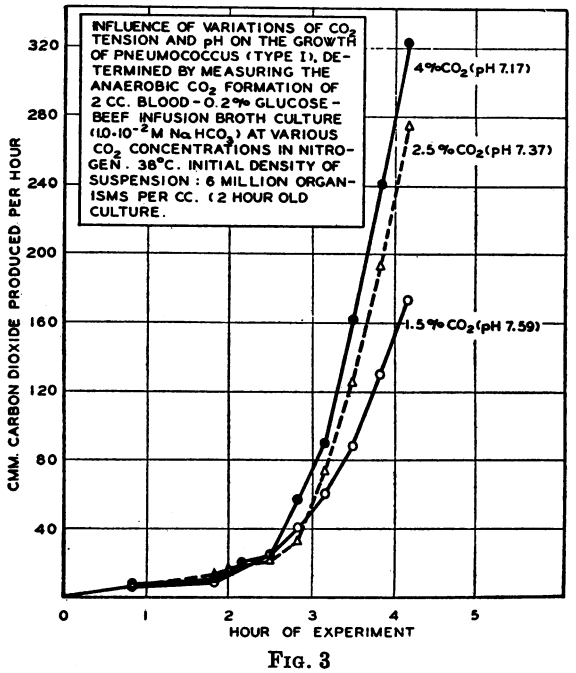

FIG. 3

SUMMARY

The effect of various CO_2 concentrations on the growth rate of pneumococci of types I, II, and III was measured manometrically with the Warburg technique.

The growth rate of the pneumococci proved to be markedly dependent upon variations of the CO_2 concentrations: in a 24-hour-old culture with relatively small inoculum (0.1 million organisms per ml.) no growth took place in 48 hours at CO_2 concentrations between 0 and 0.1 vol. per cent, whereas the logarithmic phase of multiplication was reached at 1 vol. per cent CO_2 after

41 hours, at 5 vol. per cent CO_2 after 26 hours, and at 10 vol. per cent CO_2 after only 21 hours. This growth-stimulating effect of the CO_2 as such greatly exceeds the growth-inhibiting effect caused by the more acid pH, so that even at a concentration as high as 20 vol. per cent CO_2, in spite of a more unfavorable pH, optimal growth is reached after 33 hours, i.e., 8 hours earlier than at 1 vol. per cent. The same stimulating effect of higher CO_2 concentrations on the pneumococcus growth rate, in spite of a more unfavorable pH, was found under anaerobic conditions as well, and could also be shown in fast-growing cultures with a relatively high initial density. In a 2-hour-old culture with an initial density of 6 million organisms per ml., there was, after 4 hours, an increase of 87 per cent in the number of bacteria at 4 vol. per cent CO_2 (pH 7.17) compared to that at 1.5 vol. per cent CO_2 (pH 7.59). This CO_2 effect on the growth rate of pneumococcus could be demonstrated even though the culture media contained peptone, blood, and glucose.

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