SPECIAL ARTICLES

CARBON DIOXIDE AS A FACTOR AFFECTING LAG IN BACTERIAL GROWTH

FOR many years biologists have known that inoculation of bacteria into a fresh, favorable medium is followed by a cycle of population growth and decline. Existent work on the phenomena involved has been extensively summarized in recent years by Winslow[1] and by Buchanan and Fulmer.[2] Usually the cycle includes an initial, lag period of adjustment, a period of rapid constant increase at maximum rate, a period of crisis or stable peak population, and periods of decline and readjustment.

Youth of the mother culture used to start the new population is known generally to decrease the period of lag. Transfer to a fresh medium from a culture already in active logarithmic increase in the same kind of medium often abolishes lag. Lag is also affected by the magnitude of the inoculating dose, the temperature of cultivation and the composition of both new and old media. Most theories to date regard the shortness of the adjustment phase as dependent on some aspect of the biological state of the transplanted cells. Possibilities of prior injury to the cells, of temperature shock, of variable individual cell adaptation, of growth inertia and of required development of essential secretions have been considered, Some writers have favored, as most plausible among biological state theories, the idea that bacteria, like plants, must germinate after a resting period; others have given most weight to the idea that actively growing cells contain "intermediate bodies in the synthesis of protoplasm" which are lost from old cells by diffusion; lag is caused by the time required to restore them to their essential concentration in—or around—the cells after transplantation.

Suggestion that the phenomenon of lag under certain conditions can be explained very simply as the period needed to build up the concentration of CO_2 in the environment—or in the cells—to a value essential for growth is offered in the present report.

In the course of recent studies in this laboratory on the growth curves of bacteria[3] and on metabolic activity of the cells during various population cycle phases,[4] curious influences of aeration on cultivation were noted. Continuous aeration of cultures throughout growth, by air previously freed of atmospheric CO_2 and NH_3, was found in highly nutritious media to prolong lag slightly; on the other hand, in a synthetic medium of lower nutrient value (Dolloff medium: lactose 0.5 per cent., ammonium tartrate 0.5 per cent., dibasic ammonium phosphate 0.002 per cent.) the effect of aeration was to prolong the lag period for a day or more, while unaerated test-tube cultures in the same medium grew well after a lag of not over six to eight hours. In a recent publication of these experiments,[3] the prolonged lag in Dolloff's medium resulting from aeration was interpreted as possibly due either (1) to removal by the air current of some indefinite "essential intermediate bodies in protein synthesis," as mentioned above, or (2) to removal of CO_2 necessary to growth, or (3) to a toxic effect of too much oxygenation in a poor medium.

That CO_2 in the environment is essential to cell growth had been announced as true for over one hundred bacterial strains on solid media by Valley and Rettger in 1926[5] and 1927.[6] A special series of experiments seeking to determine whether CO_2 removal or another of the three possibilities stated above was the causative factor in our results has been undertaken, and the findings are now presented.

The technique, similar to that of the previous growth curve studies,[3] involved cultivation of *Esch. coli* cultures in the Dolloff synthetic medium at 37° C, with record of growth determined at suitable frequent intervals by standard duplicate plate count methods. One purification train, for pressure aeration with CO_2-free air, was exactly as described elsewhere,[3, 7] and included among other units two spiral wash bottles containing, respectively, KOH and H_2SO_4. A second train, for aeration with air from which CO_2 was not removed, was made up from similar units, except that KOH was replaced by water.

In the first and simplest group of experiments, proportionate amounts of the same inoculating cell suspension were simultaneously introduced into three batches of Dolloff medium—one already and constantly thereafter subject to bubbling aeration with CO_2-free air, one subject to aeration with air containing atmospheric CO_2, and the third unaerated. Ammonia was, as is evident, absent from both air currents. The results of three such sets of triple culture

[1] C.-E. A. Winslow, "The Rise and Fall of Bacterial Populations." Chap. vi, pp. 58–83, in "The Newer Knowledge of Bacteriology and Immunology." Edited by Jordan and Falk. U. of Chicago Press, 1928.

[2] R. E. Buchanan and E. I. Fulmer. Chap. ii, pp. 4–62, in "Physiology and Biochemistry of Bacteria." Baltimore, Williams and Wilkins Company, 1928, Vol. I.

[3] C.-E. A. Winslow, H. H. Walker and Margaret Sutermeister, "The Influence of Aeration and of Sodium Chloride upon the Growth Curve of Bacteria in Various Media." *Jour. Bact.*, 24: 185, September, 1932.

[4] H. H. Walker and C.-E. A. Winslow, "Metabolic Activity of the Bacterial Cell at Various Phases of the Population Cycle." *Jour. Bact.*, 24: 209, September, 1932.

[5] G. Valley and L. F. Rettger, "Carbon Dioxide Requirements of Bacteria." *Jour. Bact.*, 11: 78, February, 1926.

[6] G. Valley and L. F. Rettger, "The Influence of Carbon Dioxide on Bacteria." *Jour. Bact.*, 14: 101, August, 1927.

[7] H. H. Walker, "An Aeration Train for the Study of Products of Bacterial Metabolism." *Jour. Bact.*, 24: 169, September, 1932.

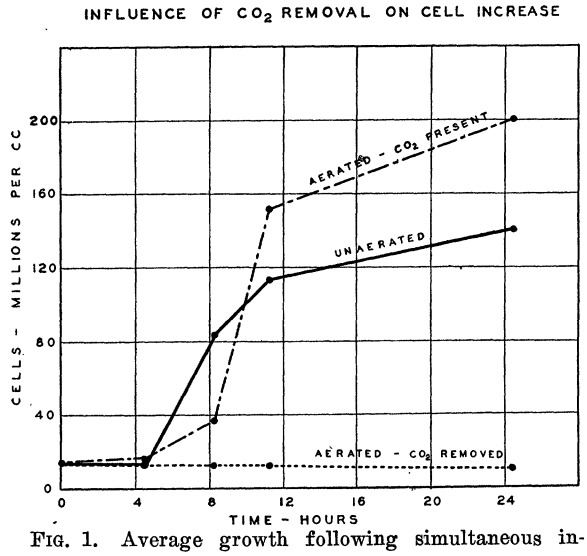

FIG. 1. Average growth following simultaneous inoculation of three portions of medium, one cultivated in a cotton plugged test-tube, one aerated continuously with CO_2-free air and one aerated at a similar rate with air from which CO_2 was not removed.

experiments, no one of which differed materially at any point from the others, have been averaged for presentation in Table 1 and are shown graphically in Fig. 1, where the coordinates represent time and cell numbers.

TABLE I

AVERAGE POPULATION GROWTH UNDER THREE AERATION CONDITIONS

Age (hours)	Millions per cc		
	Condition A	Condition B	Condition C
0	14	14	14
4–5	14	15	14
7.5–9	12	36	82
10.5–12	12	151	113
24–25	10	200	140

A—Three cultures aerated with CO_2-free air
B— " " " " CO_2-containing air
C— " " not aerated

The table and figure clearly reveal that in the unaerated medium good growth was obtained commencing after the fourth or fifth hour; aeration with air from which CO_2 had not been removed resulted in a slightly prolonged lag but gave after 12 hours even better growth; aeration with CO_2-free air permitted no growth in 24 hours. Thus are differentiated the effects of the three aeration conditions on inoculations exposed immediately to them.

Other experiments were now performed wherein three cultures started from the same inoculating suspension were first all inhibited for one day by aeration with CO_2-free air and then subjected respectively to the three conditions above. That is, after 24 hours of aeration with CO_2-free air (no growth having occurred in any bottle) aeration of one culture was discontinued, another was subjected to aeration with CO_2-containing air, and the third was continued under aeration with CO_2-free air for another day. By reference to the experiment shown in Fig. 2[8] it will be seen

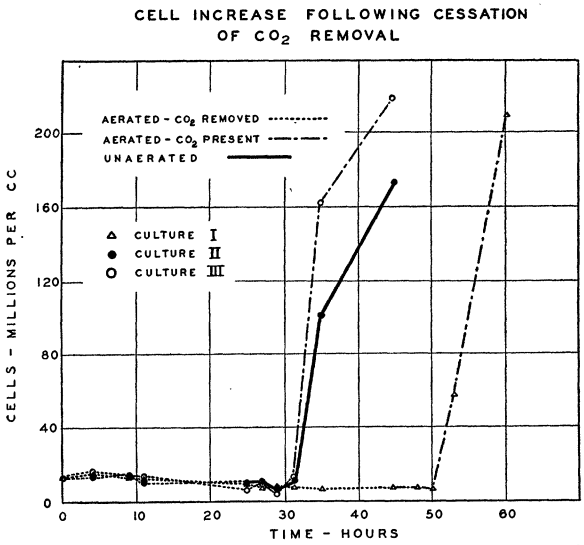

FIG. 2. Onset of growth, following discontinuance of aeration or change to aeration with CO_2-containing air, in cultures previously inhibited for one or two days by CO_2-free aeration.

Culture I aerated with CO_2-free air from inoculation to 45th hour; aerated with CO_2-containing air from 45th to 60th hour.

Culture II aerated with CO_2-free air to 25th hour; unaerated from 25th to 45th hour.

Culture III aerated with CO_2-free air to 25th hour; aerated with CO_2-containing air from 25th to 45th hour.

that the culture (I) kept on CO_2-free aeration for the second day continued its lag accordingly, but the one (II) whose aeration was stopped after the first day underwent in a few hours a normal growth period; so also did the one (III) switched after one day to an aeration train containing no KOH wash bottle (water instead). Finally, after two days of inhibition by CO_2-free aeration, the culture (I) so treated was transferred to the CO_2-containing air train and still proved itself capable of normal growth thereafter. These results appear to show clearly that cessation of removal of CO_2, from cultures previously thereby inhibited, results in normal development, no matter whether aeration is stopped entirely or CO_2 is simply no longer removed from the aerating current.

[8] In the graph, for simplicity, each curve has been continued only as far as the highest count obtained; all cultures subsequently declined in numbers.

A final experiment was performed in order to show that removal of NH_3 by the H_2SO_4 wash bottles, included up to this point in both aeration trains, was not involved in the CO_2 effects noted. To this end, H_2SO_4 was omitted from both trains; all other purification units were left as before. Five cultures were started simultaneously. Three subjected at once to CO_2-free aeration, CO_2-containing aeration, and no aeration, yielded curves similar in all respects to the corresponding ones of Fig. 1. The other two cultures were also, as in Fig. 2, subjected to inhibition by CO_2-free aeration for one day. Then one was transferred to the CO_2-containing air train and one had aeration discontinued; both grew thereafter as in Fig. 2. Likewise, one continued on CO_2-free aeration for the second day prolonged its lag accordingly, but grew on the third day, after aeration was shut off. Thus it appears that presence or absence of an H_2SO_4 wash bottle for absorption of NH_3 from the air current has no effect on the CO_2 results obtained. In short, removal or non-removal of CO_2 appears responsible for the phenomena noted.

In concluding, it may be pointed out that by the method of introducing aliquots of a common inoculating suspension into batches of the same medium subjected to the three chosen conditions—CO_2-free aeration, CO_2-containing aeration and no aeration—most of the factors claimed by earlier workers to be influential upon lag phenomena have been controlled so far as discernment of CO_2 effects requires. Items so controlled include age of mother culture, transfer of inhibitory substances therefrom, size of inoculating dose, nature of both old and new medium, prior injury to cells, temperature shock, variable cell capacity for environmental adaptation, and even particularly favored biological state effects such as the need for a germination period or restoration of postulated intermediate bodies in protoplasmic synthesis. Removal of CO_2, independent from the effect of aeration itself, is therefore the variable studied. Accordingly, we conclude that:

(1) In a synthetic medium of low nutrient value, the lag period of (coli) cultures can be prolonged at will by aeration with an air current purified by passage through a washing train which includes an alkaline, CO_2-absorbent unit.

(2) When parallel cultures are aerated at a comparable rate with air washed through a train similar in all respects, save for the replacement of the alkali by water, normal growth occurs to an even higher degree than is the case in unaerated cotton-plugged test-tube cultures in the same medium.

(3) Cultures inhibited from growth by CO_2-free aeration for a day or more will, when aeration is stopped or is resumed after replacement of the alkaline wash bottle by water, undergo a normal lag of a few hours and then grow in the manner characteristic of newly inoculated cultures in unaerated media or in media aerated with air from which CO_2 has not been removed.

(4) Presence or absence of an acid NH_3 absorbent in the air train does not alter the above phenomena.

(5) There are no differences in nutrient values of the substrate or in its reaction to account for the observed results.

(6) Aeration itself, either through mechanical agitation or through increased oxygenation of the medium, or by the washing out from the medium of metabolic products (other than CO_2), does not account for the results noted.

(7) Other factors being similar, the phenomenon must be due to the substance removed from the aeration current, hence from the medium, by the caustic wash bottle,—CO_2.

(8) Our results, then, tend to (a) Confirm in a fluid medium the necessity of CO_2 for cell growth, which Valley and Rettger demonstrated in solid media; (b) Suggest that the phenomenon of lag may be due largely, if not entirely, to the time it takes the culture to build up the CO_2 content of the medium or of the cells themselves to a value essential for growth; (c) Simplify the philosophy of the lag period by apparently rendering unnecessary the postulation of an essential formation of intermediate bodies in protein synthesis required for growth, other than CO_2.

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