THE EFFECT OF AERATION ON THE GROWTH OF AEROBACTER AEROGENES AND ESCHERICHIA COLI, WITH REFERENCE TO THE PASTEUR MECHANISM

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The Pasteur mechanism, by which fermentative activities of facultative organisms are suppressed by oxygen and are supplanted by respiratory mechanisms more efficient for growth, is a classical problem in microbiology. Further progress toward its solution would appear to depend upon an extension of our knowledge of the effects produced by air on the growth phases of facultative bacteria; for whether theories are based on oxidative inhibition of fermentation or on stimulation of aerobic processes permitting abundant growth, account must be taken of the fact that, although some workers have reported increased growth of heterotrophs, others have observed growth suppression. Thus Lwoff and Monod (1947) found that such bacteria, grown without lag in test tubes, showed prolonged lag in flasks aerated by agitation at normal CO_2 tensions, but in contrast Winslow, Walker, and Sutermeister (1932) for natural media and Lodge and Hinshelwood (1943) for synthetic showed that the stationary population (or total crop) increased considerably on aeration. Dagley, Dawes, and Morrison (1950*a*) showed that sterile filtrates from unaerated cultures of *Aerobacter aerogenes* in a glucose ammonium salt medium produce growth responses when sufficient oxygen is available; the theory that toxic products are formed in unaerated cultures, and that aeration leads to an increased crop by removing them, is therefore untenable.

In the present work we have combined investigations of the effects of aeration on the growth of *Escherichia coli* and *Aerobacter aerogenes* with determinations of pyruvate formed in cultures, since pyruvic acid occupies a key position in the fermentative activities with which the Pasteur mechanism is also concerned.

EXPERIMENTAL METHODS

The bacteria used were N.C.T.C. strains: *Aerobacter aerogenes* no. 418 and *Escherichia coli* no. 5928. The methods used for obtaining growth curves and the precautions taken when growing cultures have been described previously (Dagley, Dawes, and Morrison, 1950*b*). The following solutions were used: (A), 9 g KH_2PO_4 per liter of glass-distilled water, pH adjusted to 7.1; (B), as for (A) with the addition of 2 g $(NH_4)_2SO_4$ per liter; (C), 10 g $MgSO_4 \cdot 7H_2O$ per liter. These, and the glucose solutions used, were sterilized separately. A suitable growth medium, in which bacteria were prepared for any series of experiments by three serial subcultures, was made up by dispensing aseptically into sterile boiling tubes 15 ml of (B), 10 ml of a solution of 30 g glucose per liter, and 0.1 ml of (C). Cultures were grown at 39 C in three ways: (1) with the medium aerated

by a gentle stream of sterile air; (2) not aerated, but with normal access of air by diffusion; (3) in a McIntosh and Fildes jar, the usual precautions being taken to ensure anaerobic conditions. When stationary populations were obtained by method 3, growth curves were not constructed, but the cultures were sampled after 18 hours, when experience showed that cell division had ceased.

The amounts of pyruvic acid present in growth supernatants were estimated by the toluene extraction method of Friedemann and Haugen (1943). A calibration curve was prepared with freshly distilled pyruvic acid using a Spekker photoelectric absorptiometer with Ilford filters (neutral H508 and green OG1).

RESULTS

Dependence of stationary population on concentration of glucose or ammonium salt. In synthetic media the logarithmic phase is succeeded by a stationary phase for which the bacterial population is related linearly to concentration for particular substrates (Dagley and Hinshelwood, 1938; Monod, 1942). We have investigated these relationships for *A. aerogenes* under the growth conditions specified, for glucose and ammonium sulfate. In the first series of experiments, media contained 15 ml of solution (B), 0.1 ml of (C), and 10 ml of various solutions of glucose to provide a concentration range 0 to 3×10^{-2} M glucose; and in the second series 15 ml of (A), 0.1 ml of (C), 2.5 ml of a solution of 40 g glucose per liter, and 7.5 ml of various solutions of $(NH_4)_2SO_4$ providing a range 0 to 3×10^{-3} M $(NH_4)_2SO_4$. Previous work had indicated that for unaerated cultures ammonium salt would be in excess for the first series and glucose for the second; the respective concentrations are 9×10^{-3} M $(NH_4)_2SO_4$ and 2.2×10^{-2} M glucose. In figure 1 stationary populations of bacteria are plotted against substrate concentrations.

It is seen that the linear relation between substrate concentration and crop breaks down for anaerobic and unaerated cultures at 8.6×10^{-3} for glucose, $(NH_4)_2SO_4$ being present initially at 9×10^{-3} M, and at 1.3×10^{-3} M $(NH_4)_2SO_4$ with initial glucose at 2.2×10^{-2} M. This indicates that in the region where the curves lie parallel to the axis, both glucose and $(NH_4)_2SO_4$ are present in excess in both series. This was verified by estimation of the glucose by the method of Somogyi (1937) both for the ascending and horizontal sections of the curves. Where glucose was limiting, none could be detected in stationary phase supernatants, but for those beyond the range of linear increases excess glucose was present. Similarly, tests with Nessler reagent demonstrated the presence of excess ammonia for cultures beyond the range of linear increase. It is clear, therefore, that in this range cell division ceases in the presence of excess of both substrates; and since it commences again on aeration and does not cease until a stationary population corresponding to that of an aerated culture is given, growth was not stopped because of the development of an adverse pH. It is also seen that in the range of linear increase aeration has little effect upon the stationary population when ammonium salt is limiting, whereas in the corresponding range for glucose the crop is more than doubled.

Pyruvic acid and growth. The importance of pyruvic acid for cell division is

illustrated by the following experiment. Ten ml of a washed suspension of *A. aerogenes* that had been grown with aeration in glucose ammonium salt medium was added to a mixture of 60 ml of solution (A), 0.4 ml of (C), and 30 ml of a solution of 10 g glucose per liter in a large (8 by 1½ inches) boiling tube. A gentle stream of sterile air was passed through the culture, which was incubated at 39 C. At intervals, 5-ml samples were withdrawn and rapidly centrifuged, and the pyruvate concentration was determined in 3 ml of supernatant. After 3

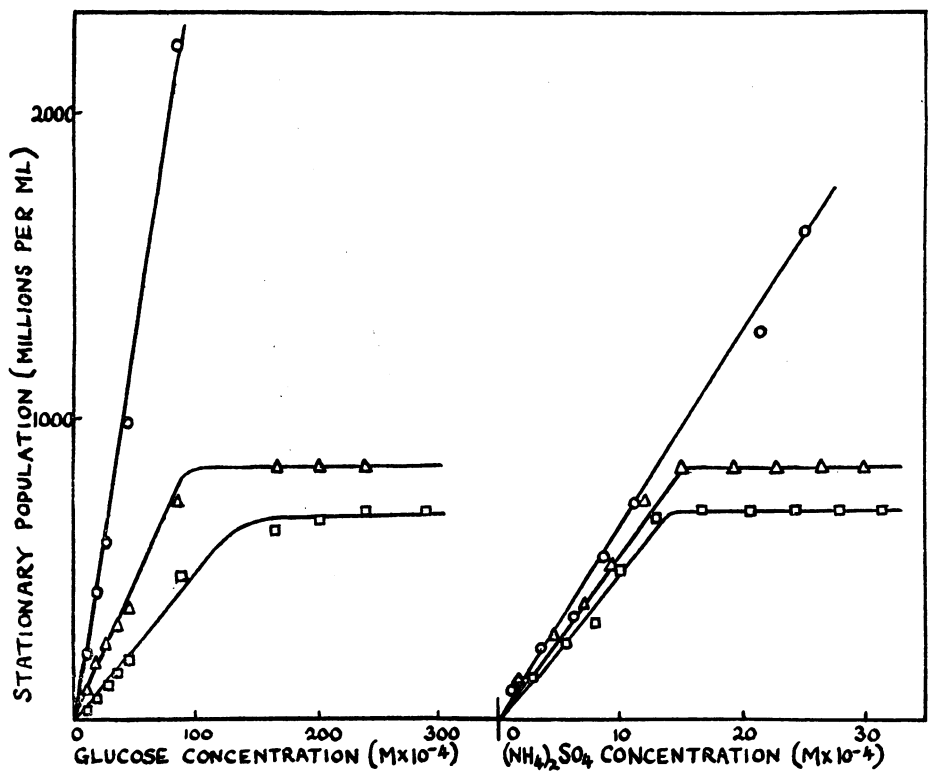

Figure 1. The relation between substrate concentration and a stationary population of *A. aerogenes*. Cultures grown with aeration, ○; with no aeration, △; strictly anaerobically, □.

hours the growth medium was completed by the addition of sufficient $(NH_4)_2SO_4$ to give a concentration of 1 g per liter, and pyruvate determinations were continued, together with measurements of the bacterial population.

Figure 2 shows an initial linear increase in pyruvate, but this is immediately arrested when the source of nitrogen is added and growth commences. Later, pyruvate production by the increasing cell population is more than adequate for growth needs, and further accumulation occurs. Work in progress in this laboratory shows that the ability of cells to grow on other sources of carbon, nitrogen being supplied as ammonium salt, depends on their ability to produce pyruvic acid from the media.

Dual effect of aeration: relation to pyruvate production. The effect of aeration is not confined to favoring growth by increasing stationary populations, for brisk aeration, particularly of light inocula, may produce a lag period. A medium containing 60 ml of solution (B), 0.4 ml of (C), and 40 ml of a solution of 30 g

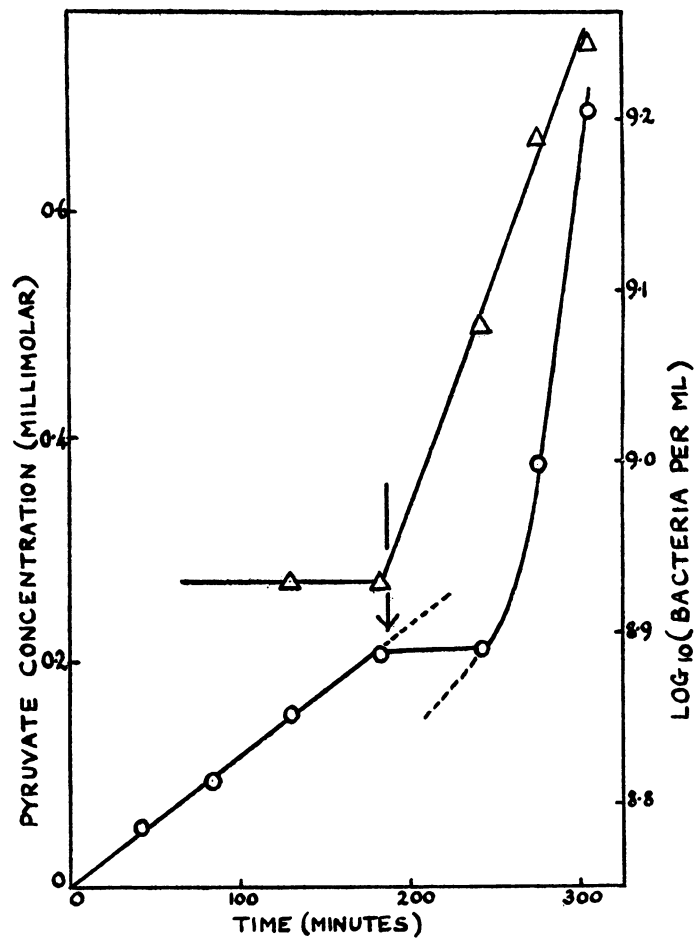

Figure 2. Effects on growth and pyruvic acid production of adding ammonium sulfate to a suspension of *A. aerogenes* gently aerated. The time of addition is shown by an arrow. Pyruvate concentration, O; growth, △.

glucose per liter was inoculated with *A. aerogenes* and after incubation without aeration reached a stationary phase with nutrients in excess, as previously described. Two hours later it was vigorously aerated, and changes in pyruvate concentration and bacterial population were followed.

Figure 3 shows an immediate rise in pyruvate concentration on aeration, but cell division lags for about 1 hour. Evidently this growth inhibition cannot be attributed to a blockage of reactions producing pyruvate. When the logarithmic

phase begins, the rise in pyruvate concentration is arrested, as was the case in figure 2, but as growth proceeds pyruvic acid again accumulates. It will be observed that pyruvate concentration, for the culture in its unaerated stationary phase, remained practically constant. We have previously shown (Dagley, Dawes, and Morrison, 1950*c*) that pyruvic acid disappears much more rapidly from aerated than from unaerated cultures in their stationary phases. Cultures grown under strictly anaerobic conditions to obtain data for figure 1 also con-

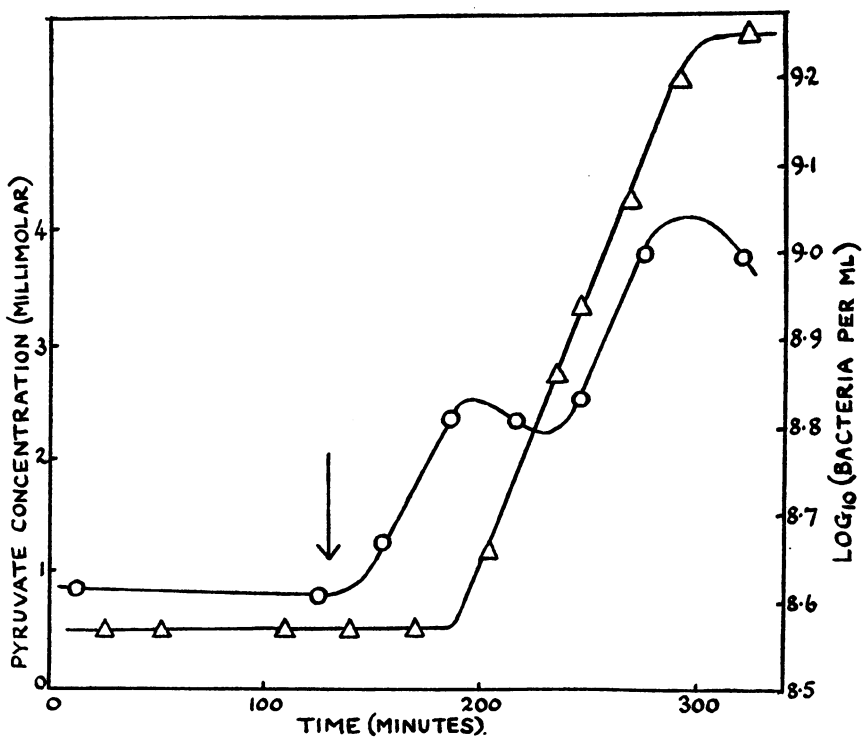

Figure 3. Effects on growth and pyruvic acid production when a culture of *A. aerogenes* is vigorously aerated after reaching the stationary phase unaerated. The time of aeration is shown by an arrow. Pyruvate concentration, O; growth, △.

tained pyruvic acid when this phase was reached in the presence of excess nutrients. Production of pyruvic acid in cultures of *E. coli* when aerated sufficiently vigorously to inhibit growth was also demonstrated. Our strain of this organism was rather more sensitive to aeration than the *A. aerogenes*, and lags of several hours sometimes occurred prior to the aerated logarithmic phase.

Origin of pyruvic acid. It is well established that various organic acids accumulate in glucose cultures, and the possibility therefore arises that the sudden increase in pyruvate concentration on the aeration of a culture stationary in the presence of excess nutrients is not primarily due to the resumption of glycolysis. The results of an experiment in this connection are shown in figure 4. *A. aerogenes* was allowed to reach its unaerated stationary phase in two media, for one of

which glucose was limiting at 8×10^{-3} M initially, and for the other, in excess of requirements, at 2×10^{-2} M initially. On aeration, a very small increase in pyruvate concentration occurred in the first culture and a large increase in the second; a resumption of growth occurred in both cases, the stationary phase setting in at a time corresponding to the maximum in figure 4 for the culture with excess glucose, which also reached a higher stationary population. Clearly

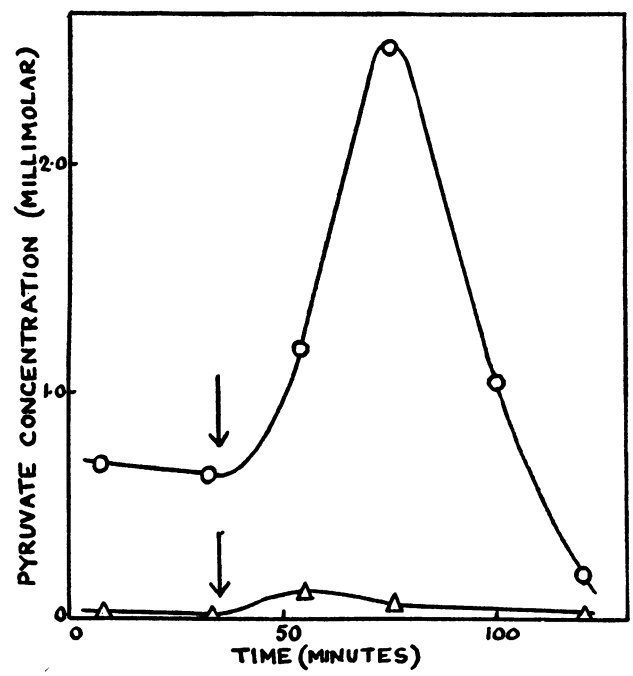

Figure 4. Dependence of pyruvic acid production on the presence of glucose. Cultures of *A. aerogenes* were aerated at the time shown by arrows, after reaching the stationary phase without aeration, in the presence of excess glucose, O, and limiting concentration of glucose, △.

the pyruvic acid produced arises primarily from glucose rather than from by-products.

DISCUSSION

In unaerated and anaerobic cultures, with glucose in excess of growth requirements, the relationship between stationary population and added ammonium salt is linear up to 1.3×10^{-3} M, but further additions produce no increase in crop. Similarly with excess ammonium salt the stationary population increases with glucose concentration up to 8.6×10^{-3} M and then remains at the maximum value reached by ammonium salt additions to excess glucose. We have confirmed by tests for glucose and ammonia that both substrates may be present in excess when this maximum stationary population is reached. Filtrates from unaerated cultures in glucose ammonium salt media do not contain appreciable amounts of

toxic products of metabolism; on the contrary, they promote growth at suitable oxygen tensions (Dagley, Dawes, and Morrison, 1950*a*); neither can the failure of substrate additions to increase the crop be attributed to the development of an adverse pH, since growth is resumed on aeration. These results would, however, accord with the view that the attainment of maximum stationary population corresponds to the complete conversion of cozymase to its reduced form, with the consequent inability of glycolysis to proceed beyond the stage of phosphoglyceraldehyde and provide pyruvic acid at a rate adequate for the heavy demands of logarithmic growth. Meyerhof (1942) has pointed out that one atom of oxygen inhibits the formation of one molecule of ethanol in fermentation and has suggested that, when oxygen is admitted, acetaldehyde (or pyruvic acid) is not reduced by dihydrocozymase but oxygen is reduced to water instead. It has been objected (Stephenson, 1949) that this explanation sheds no light on the fate of pyruvic acid, which does not accumulate when growth occurs instead of fermentation. The results we have recorded here and elsewhere (Dagley, Dawes, and Morrison, J. Gen. Microbiol., *in press*) prove, however, that pyruvate is a transitory intermediate in growth processes and rapidly disappears in aerated cultures when the stationary phase is reached; moreover, it is present in stationary anaerobic cultures, suggesting that its rate of conversion to fermentation products is considerably less than its rate of utilization in growth. The attainment of an almost stationary level of pyruvic acid in anaerobic conditions, concomitant with growth cessation, is consistent with the view that interruption of exergonic glycolytic reactions following the triosephosphate stage deprives the cell of the free energy required for the assimilation of pyruvic acid and the essential metabolites to which it gives rise.

The rapid production of pyruvic acid when cultures containing excess glucose are aerated, but not when growth has exhausted glucose, accords with the view that growth in unaerated cultures ceases because of the interruption of glycolytic formation of pyruvate, as a consequence of the absence of oxygen as a ready hydrogen acceptor. Theories attributing the Pasteur mechanism to a positive inhibition by oxygen are not compatible with our results. We have shown that very vigorous aeration of cultures may inhibit cell division, but pyruvate production is not affected; impairment of pyruvate assimilation by the cells presumably occurs, due possibly to the difficulty of aminating pyruvic and other keto acids under these conditions since, as we have shown (Dagley, Dawes, and Morrison, 1950*b*,*c*), the production of amino acids precedes growth in the medium used in these experiments.

When the stationary population is limited by glucose concentration, a given amount of glucose will support considerably more growth in an aerated than in an unaerated medium; whereas, when ammonium salt is limiting, aeration does not affect cell population. This would appear to be a consequence of the fact that the function of ammonia is to provide cellular material, whereas glucose is, in addition, a source of energy. In unaerated cultures compounds such as succinic acid accumulate which only support growth on aeration (Dagley, Dawes, and Morrison, 1950*c*) because of their inability to liberate energy for growth in

anaerobic conditions. It is clear, therefore, that the effect of oxygen is not entirely confined to providing a hydrogen acceptor for dihydrogen cozymase but may control the utilization of substrates in other ways. Working with unaerated cultures (Dagley, Dawes, and Morrison, 1950*a*,*b*), we have shown that additions of dicarboxylic and amino acids to a glucose ammonium salt medium produce growth responses dependent on the tension of dissolved oxygen, and also that fumaric and malic acids were inhibitory in contrast to succinic, which was stimulating; but Baskett and Hinshelwood (1950), who used aerated cultures, did not observe inhibition with fumarate and malate additions.

SUMMARY

For anaerobic and semianaerobic cultures of *Aerobacter aerogenes* and *Escherichia coli* in glucose ammonium salt media, the linear relation between stationary population and concentration breaks down at 8.6×10^{-3} M glucose and 1.3×10^{-3} M ammonium sulfate. No increase in crop results from further nutrient additions; cell division ceases with substrates unconsumed but is resumed on gentle aeration. Growth is delayed if aeration is very vigorous, and there is rapid accumulation of pyruvic acid in the culture. Evidence is produced to show that this is due to a resumption of glycolysis and consequent production of pyruvate at a greater rate than its utilization in growth. The bearing of these observations on theories of the Pasteur mechanism is discussed. When the supply of ammonium sulfate limits the total growth, the stationary population is approximately the same for aerated as for unaerated cultures, but a given limiting amount of glucose will support a considerably higher cell population if the culture is aerated.

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